Environmental Research 168 (2019) 467–489

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Review article

Microcystin-RR: Occurrence, content in water and food and toxicological studies. A review

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ARTICLE INFO

Keywords:
Cyanotoxins
Microcystin
MC-RR
Occurrence
Toxicity

ABSTRACT

Microcystins (MCs) are hepatotoxins, produced by various species of cyanobacteria, whose occurrence is increasing worldwide owing to climate change and anthropogenic activities. More than 100 variants have been reported, and among them MC-LR is the most extensively studied, but there are other MC congeners that deserve to be investigated. The need for data to characterize the toxicological profile of MC variants other than MC-LR has been identified in order to improve risk assessment in humans and wildlife. Accordingly, the aim of this study was to evaluate the information available in the scientific literature dealing with MC-RR, as this congener is the second most common cyanotoxin in the environment. The review focuses on aspects such as occurrence in water and food, and toxicity studies both in vitro and in vivo. It reveals that, although MC-RR is a real hazard with a high exposure potential in some countries, little is known yet about its specific toxicological properties that differ from those of MC-LR, and important aspects such as genotoxicity and chronic effects have not yet been sufficiently addressed.

1. Introduction

Microcystins (MCs) are a well known group of cyanobacteria toxins. These cyanobacteria are photosynthetic prokaryotes mainly present in freshwater ecosystems, although they can also be found in other environments (Testai et al., 2016). Cyanotoxins are generally produced as a consequence of the increasingly frequent appearance of cyanobacteria blooms due to climate changes and anthropogenic activities (Buratti et al., 2017). The main freshwater cyanobacteria that can produce MCs include species from the following genera: *Microcystis*, *Anabaena*, *Aphanizomenon*, *Dolichospermum*, *Limnothrix*, *Nostoc*, *Phormidium*, and *Planktothrix* (Catherine et al., 2017). Moreover, the occurrence of these MC-producing cyanobacteria has now been reported worldwide (Catherine et al., 2017), and so have the toxic effects of cyanotoxins on humans and wildlife (Briand et al., 2003; Zurawell et al., 2005; Pichardo et al., 2007; Buratti et al., 2017). On the basis of their toxin production, to which humans can be exposed via various routes, the World Health Organization (WHO) has listed cyanobacteria as an emerging health issue (Testai et al., 2016).

MCs are mainly intracellular toxins with typically less than 30% of the total concentration being extracellular (Graham et al., 2010). However, cell lysis due to bloom collapse or human activities can increase the extracellular fraction level (Sivonen and Jones, 1999). MCs have the following cyclic heptapeptide structure: (D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda-Arg^5-D-Glu^6-Mdha^7), where X and Z are variable amino acids, D-MeAsp is D-erythro-β-methylaspartic acid, Adda is (2 S, 3 S, 8 S, 9 S)-3-amino-9-methoxy-2–6–8-trimethyl 10-phenyldeca-4,6-dienoic acid, unique for cyanobacteria, and Mdha is N-methyldehydroalanine. The L-amino acid residues at positions 2 and 4 account for many of the MC variants. As a result of this, together with common variations in the general structure (i.e. demethylations in residues 3 and 7, etc.), more than 100 variants have now been reported (Buratti et al., 2017). In fact, the number of known MC congeners has increased rapidly in recent years, thanks to more sensitive mass spectrometric techniques (a recent updated list can be found in Spoof and Catherine, 2017). Among all of them, MC-LR, containing leucine (L) at position 2 and arginine (R) at position 4, is the most extensively studied, owing to its toxicity, ubiquity, and prevalence.

MCs are generally classified as hepatotoxins as it is well established that the main target of MCs in mammals is the liver, primarily because of toxin update by the bile acid transport system (Hermansky and Stohs, 1991; Tencalla et al., 1994). However, depending on the specific structure of the congeners and their differential toxicokinetic profiles, other target organs have also been reported (Amé et al., 2010).

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https://doi.org/10.1016/j.envres.2018.07.019
Received 24 April 2018; Received in revised form 9 July 2018; Accepted 9 July 2018
Available online 12 July 2018
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On the basis of their toxicity and their presence in water and food, the main exposure pathways for humans, various health-based guidance values for MCs have been derived. The WHO established a guideline value for drinking water of 1 μg/L MC-LR and the tolerable daily intake (TDI) value for a 60 kg adult was set at 0.04 μg/kg body weight/day (Chorus and Bartram, 1999). No specific guidance values have been defined for other congeners. Moreover, risk assessment is performed on the basis of MC-LR, and when there are other congeners involved data are given as MC-LR equivalents.

Nevertheless, the investigation of other MC variants is of importance because MCs usually occur as mixtures of different variants, and also because the scarce number of reports on other congeners may leave the mistaken impression that they are of minor concern in relation to human health (Testai et al., 2016). In this regard, more data on the characterization of the toxicological profiles of different MC congeners are needed (Testai et al., 2016). Moreover, several authors, such as Hoeger et al. (2007), Dietrich and Hoeger (2005), and Faassen and Lürling (2013), have suggested that MC congeners can differ profoundly in their toxicodynamic and -kinetic properties, and therefore risk assessment schemes based on MC-LR equivalents are insufficient.

In the present work the focus is on MC-RR, which is a MC variant with the amino acid arginine in positions 2 and 4 (Fig. 1). This structural characteristic makes this congener more hydrophilic (polar) than other variants, and this plays an important role in its toxicokinetics. MC-RR is the second most extensively studied MC variant after MC-LR. This is also due to its frequent occurrence in the environment and the availability of commercial standards that allow its chemical detection, determination, and quantification. Therefore, the aim of this review was to compile the information available in the scientific literature dealing with MC-RR, with a special focus on 1) occurrence in water and food, and 2) toxicity studies, both in vitro and in vivo. The analysis of the data will make it possible to obtain a better characterization of the toxicological profile of MC-RR, to establish the suitability of a specific risk assessment for this particular MC congener, and also to identify potential data gaps that require further study.

2. Occurrence and content in water and food

2.1. MC-RR in water samples

MC-producing cyanobacteria such as *Anabaena*, *Dolichospermum*, *Microcystis*, or *Planktothrix* have a cosmopolitan distribution, with blooms in more than 80 countries (Zurawell et al., 2005; Catherine et al., 2017), and the same behavior has been observed for the occurrence of the cyanotoxin MC-RR. The analysis of the scientific literature (2010–2018) shows that the presence of MC-RR in water has been reported around the world, and the most noteworthy results concerning the detection of this toxin are presented in Table 1, which has details of the studies where this specific MC congener has been detected and quantified.

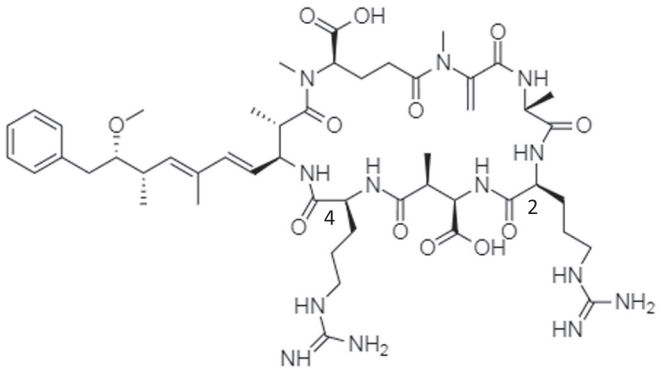

Fig. 1. Chemical structure of MC-RR.

In most of the articles reviewed the concentrations of MCs reported in water were lower than the WHO guideline value (1.0 μg/L for MC-LR); however, the MC concentrations in cyanobacterial cells were too high (Jia et al., 2016). In this regard, Li et al. (2010) reported that the intracellular MC-RR ranged between 15% and 95% of total MCs, depending on the abundance and concentration of *Microcystis* spp. and the stage of the bloom over the period studied, while more than 90% of the cyanotoxins were intracellular during the bloom period. This means that free dissolved MC-RR could be released and remain in water, or could be released during water treatment processes, increasing human health risks (Jia et al., 2016). Consequently, bioaccumulation of MCs in fish and plant tissues owing to their high extracellular concentration should be taken into account (Mbukwa et al., 2012).

Many new case studies on MC-producing cyanobacteria present in water and blooms have been published in the last decade (Catherine et al., 2017). Since 2010, MC-RR has been found in water bodies and cyanobacterial blooms in Africa, America, Asia, and Europe, and is the most frequent MC congener in lakes, rivers, and reservoirs in China (Junfeng et al., 2010).

There are a few reports that deal with the presence of MC-RR in Africa; although, this continent is particularly vulnerable to cyanotoxins due to eutrophication of many water bodies (Preece et al., 2017). MC-RR was the major congener found in Moroccan and Algerian lakes (Douma et al., 2010; Bouhaddada et al., 2016). Moreover, MC-RR and dm-MC-RR ([D-Asp^{3}Dha^{7}MC-RR]) were found as major and minor MC variants in South Africa (Mbukwa et al., 2012). Moreover, these authors reported that concentration of these MC in fish reached the detrimental range. By contrast, while MC-RR was found in more than 10% of samples in Lake Victoria (Tanzania), the congener MC-LY was found as the dominant MC in this lake (Miles et al., 2013).

Similarly, the occurrence of MC-RR in America has been scarcely been studied, and the presence of this congener in water is usually associated with MC-LR, the most common MC in environmental waters (Graham et al., 2010). Zastepa et al. (2014) reported that MC-RR was found in Canadian waters as a minor congener at low concentrations, while MC-LA was the dominant variant present in blooms. In South America, the presence of MC-RR has been detected in Argentina (Amé et al., 2010) and differences were found in MC-RR concentrations during the wet (90.7%) and dry (0%) seasons. In this regard, distribution and composition of cyanobacterial blooms are related to a great variety of parameters.

Apart from Vietnam and India, China is the Asian country conducting more studies were between 2010 and 2018: these have revealed the presence of MC-RR in water bodies. In China, the growth of algal blooms is ensured by the rapid eutrophication of Chinese lakes, associated with the pollution produce by fast economic development and intensive use of water sources (Peng et al., 2010). Although MC-LR being considered the most frequent MC variant in the environment (Srivastava et al., 2012), MC-RR is the major toxin variant found in Asian lakes and rivers (Jia et al., 2016; Li et al., 2010; Yen et al., 2012; Singh et al., 2015; He et al., 2018; Srivastava et al., 2012), reaching concentrations of up to 93.5% in cyanobacterial cells (Liu et al., 2011). At molecular level, the presence of microcystin synthetase gene, mcyB, was found to correlate well with MC-RR concentrations determined in some reservoirs, in agreement with the predominance of this toxin (Yen et al., 2012). Although MC-RR is less toxic than MC-LR (Dittmann and Wiegand, 2006; He et al., 2018), the very high concentrations found in some lakes should be of concern with regard to human risks.

Some authors have related the seasonal distribution of MC to water currents or to the strains present in blooms, depending on suitable water temperatures, adequate nutrients, and illumination (He et al., 2018; Zhang et al., 2017). In this regard, it has been suggested that low levels of MC-RR (and MC-LR) during the dry season could be due to a combination of low levels of available nutrients, relatively low phytoplankton density, and low water temperatures; moreover, suspended particulate matter plays an important role in reduction of MC (He et al.,

Table 1
Presence of MC-RR in water samples.

Country	Location	Sample date	Congeners identified	Analytical method	Reference
Africa	Morocco (two Moroccan lake reservoirs)	2004	MC-LR, **MC-RR**, MC-YR, MC-FR, MC-*WR*	HPLC-DAD, LC-MS	Douma et al. (2010)
	Tunisia (cyanobacterial strains isolated from seven Tunisian reservoirs)	-	MC-LR, MC-FR, **MC-RR**, MC-YR, MC-*WR*, Demethyl-MC-LR, Demethyl-MC-YR, Demethyl-MC-RR	MALDI-TOF	Fathalli et al. (2011)
	South Africa (Hartbeespoort Dam)	2010–2011	MC-LR, **MC-RR**, MC-YR, MC-*WR*, MC-(H_4)YR, Demethyl-MC-RR	LC-ESI-MS	Mbukwa et al. (2012)
	Tanzania (Lake Victoria)	2010	MC-LR, MC-YR, **MC-RR**, MC-LA, MC-LF, MC-LY, MC-LW, MC-RY, MC-RL, MC-RA	LC-HRMS, LC-MS/MS NMR spectroscopy	Miles et al. (2013)
	Algeria (Lake des Oiseaux)	2013	**MC-RR (43.4%)**, MC-LR (13.1%), MC-FR (9.4%), MC-YR (5.4%), MC-*WR* (7.4%)	LC-MS/MS, PPIA	Bouhaddada et al. (2016)
America	Argentina (Los Padres Lake)	2007	**MC-RR** (celullar: 1.30 μg/L; dissolved: 1.50 μg/L), MC-LA, MC-LR, MC-YR	HPLC-MS/MS	Amé et al. (2010)
	United States (23 eutrophic lakes and reservoirs)	2006	MC-LA, MC-LF, MC-LR, MC-LW, MC-LY, **MC-RR** (0.03–16000 μg/L), MC-YR	LC-MS/MS	Graham et al. (2010)
	Lake Pinto (EEUU)	2007	MC-LA, **MC-RR**, MC-LR, MC-dLR, MC-LF, MC-YR	LC-MS/MS	Miller et al. (2010)
	Canada (Ottawa)	2009–2010	MC-LA, **MC-RR** (0.2 μg/L)	HPLC-DAD	Zastepa et al. (2014)
Asia	China (Lake Chao)	2007	MC-LR, **MC-RR**, MC-YR, MC-LA, MC-LF, MC-LW and demethylated toxins (dm-MCs) and even double dm-MCs	LC-ESI-MS/MS	Krüger et al. (2010)
	China (Yanghe reservoir)	2007	**MC-RR** (dissolved: 1.56 μg/L intracellular: 70.1 μg/L), MC-YR, MC-LR	HPLC-MS/MS	Li et al. (2010)
	China (3 lakes)	–	MC-LR, **MC-RR** (0.22–1.10 mg/g intracellular)	HPLC-UV	Peng et al. (2010)
	China (Lake Taihu)	–	MCs (35 ng/L in water). MC-LR, **MC-RR** (93.5% intracellular), MC-YR	HPLC-UV (algal cells)	Liu et al. (2011)
	Taiwan (China): eutrophic lakes	2007	MC-LR, **MC-RR** (50–790 ng/L), MC-YR, MC-LW, MC-LF, MC-LA	LC-ESI-MS	Yen et al. (2011)
	India (Varanasi)	2010–2011	MC-LR, **MC-RR** (49.85%), MC-YR	LC-MS	Srivastava et al. (2012)
	China (Taiwan)	2003–2007	MC-LR, **MC-RR**, MC-YR, MC-LW	LC-MS	Yen et al. (2012)
	Vietnam (Nui Coc reservoir)	2009–2010	MC-LR, **MC-dmRR**	HPLC-MS	Duong et al. (2013)
	China (Huai River Basin)	2008–2009	MC-LR, **MC-RR** (4–19%), MC-YR	HPLC-DAD	Tian et al. (2013)
	China (Lake Erhai)	2010	MC-LR, **MC-RR** (0.002–0.748 μg/L)	HPLC-DAD	Yu et al. (2014)
	India (two freshwater ponds)	2010–2011	MC-LR, **MC-RR** (60–64%), MC-YR	LC-MS	Singh et al. (2015)
	China (Poyang Lake)	2012	**MC-RR** (intracellular MCs: 94.70%; extracellular: 84.73%), MC-YR, MC-LR, MC-LA, MC-LF, MC-LW	UPLC-MS/MS	Zhang et al. (2015)
	China (Lake Taihu)	2013–2014	MC-LR, **MC-RR** (35.1–111.5 ng/L), MC-YR	HPLC-MS/MS	Jia et al. (2016)
	China (Zhejiang, aquaculture pond)	2015	MC-LR, **MC-RR** (4–4.7 μg/L)	LC-MS/MS	Hu et al. (2017)
	China (Poyang Lake)	2013	**MC-RR** (extracellular: up to 162.37 ng/L; intracellular: up to 8067.09 ng/L), MC-YR, MC-LR	UPLC-MS/MS	Zhang et al. (2017)
	China (Yulin river)	2016	MC-LR, **MC-RR** (non-detectable (ND) − 3.56 μg/L)	HPLC-UV	He et al. (2018)
	China (Lake Chaohu)	2011–2012	MC-LR, **MC-RR** (extracellular: up to 2.45 μg/L intracellular: up to 1.47 μg/L), MC-YR	HPLC-DAD	Shang et al. (2018)
Europe	Greece (lakes)	–	**MC-RR** (0.005–0.060 μg/L), MC-LR, MC-YR	HPLC-PDA, LC-MS/MS	Triantis et al. (2010)
	Ireland (14 sites)	2008	**MC-RR** (2.4–1510 ngMC/μgChla), MC-YR, MC-LR, MC-LY, MC-LF	LC-MS/MS	Mooney et al. (2011)
	The Netherlands (surface waters)	–	MC-LR, MC-YR, dm−7-MC-LR, **MC-RR, dm−7-MC-RR**, MC-LY, MC-LW, MC-LF	LC-MS/MS	Faassen and Lürling (2013)
	Italy (Lake Occhito)	2009	MC-RR **(0.6–1.2 ng/L),** desMeMC-RR	MALDI-TOF-MS, LC/ESI-q/TOF-MS/MS	Ferranti et al. (2013)
	Poland (Great Mazurian Lakes)	2007	MC-LR, **MC-RR** (0.003–0.048 μg/L), MC-YR	HPLC-MS	Jakubowska et al. (2013)
	Greece (Lake Marathonas, Athens)	2007–2010	MC-LR, **MC-RR** (2–174 ng/L), MC-YR, MC-LA	LC-ESI-MS/MS	Kaloudis et al. (2013)
	Portugal (Alqueva and Beliche water reservoirs)	2011	**MC-RR** (14–212 ng/L), MC-YR, MC-LR	LC-ESI/Ion trap-MS/MS (negative ionization mode)	Rodrigues et al. (2013)
	Hungary (lake)	–	[D-Asp^3, Mdha^7]MC-RR	HPLC-DAD, MALDI-TOF	Vasas et al. (2013)
	Germany (Neukirchener Lake)	2013–2014	MC-LR, **MC-RR**	UPLC-MS/MS	Rodriguez et al. (2014)
	Bulgaria	2012–2014	MC-LR, **MC-RR** (0.07–103.2 μg/L), MC-YR		Pavlova et al. (2015)
	Sweden (lakes and ponds)	–	MC-LR, **MC-RR** (0.52–40.35 μg/L), MC-YR, MC-LF, MC-LW	UPLC-MS/MS	Pekar et al. (2016)
	England (70 water bodies)	2016	MC-LR, **MC-RR (≈30%)**, MC-YR. MC-LA, MC-LF, MC-*WR*, D-Asp3-MC-LR, MC-HtyR, MC-LY, MC-LW, MC-HilR, D-Asp3-MC-RR	LC-MS/MS	Turner et al. (2018)

Abbreviations: HPLC-DAD or HPLC-PDA: High performance liquid chromatography-photodiode array detector; LC-ESI-MS/MS. Liquid chromatography—electrospray ionization - tandem mass spectrometry; LC-HRMS: High-resolution LC-MS; MALDI-TOF: Matrix assisted laser desorption ionization-time of flight spectroscopy; NMR: Nuclear magnetic resonance; PPIA: Protein phosphatase inhibition assay; UPLC-MS/MS: ultra-light liquid chromatography-electrospray ionization tandem triple quadrupole/mass spectrometry.

Table 2
Presence of MC-RR in food.

Species (Common name)	MC-RR concentration (ng/g d.w.)	Location (Country, site)	Analytical method	Reference
BIVALVES				
Sinanodonta woodiana	M: approx. up to 20,000; D: approx. up to 200,000 M: approx. up to 30,000; D: approx. up to 300,000 M: approx. up to 25,000; D: approx. up to 220,000	Korea	HPLC-UV	Kim et al. (2017)
Sinanodonta arcaeformis				
Unio douglasiae				
CRUSTACEAN				
Procambarus clarkii	S: 4180; I: 730; Gn: 400; G: 130	China	LC-MS	Chen and Xie (2005)
Procambarus clarkii	WB: ND − 39.1; ND − 7.8 b.w.	Spain (Cáceres)	LC-MS/MS	Ríos et al. (2013)
FISH				
Odontesthes bonariensis	L: ND − 49.2 ± 15.8; M: 1.6 ± 1.0–3.0 ± 2.6	Argentina (Los Padres Lake)	HPLC-MS/MS	Amé et al. (2010)
Oreochromis niloticus (Nile tilapia)	I > L > M	Uganda (Lakes)	LC-MS	Semyalo et al. (2010)
Aristichthys nobilis (Bighead carp)	L: 13–43	Laboratory doses: 0.01, 0.05, 0.25 g spiked in 50 mg of tissue	LC-ESI-MS/MS	Wu et al. (2010)
Carassius auratus (Crucian carp)	K: 13 − 88	Laboratory doses: 150 and 600 MC-LR and MC-RR b.w.	LC-MS	Li et al. (2013)
Cyprinus carpio (Common carp)	L > K > M	China (Lake Taihu)	LC-MS/MS	Wu et al. (2013)
Hypophthalmichthys molitrix (Silver carp)	M: up to 36	China	LC/MS	Zhang et al. (2013)
Hypophthalmichthys molitrix (Silver carp)	M: 20.3 ± 0.766; L: 23.3 ± 1.51; K: 30.0 ± 10.1; I: 220 ± 172; H: 26.3 ± 22.6 M: 19.6 ± 0.51; L: 19.9 ± 2.05; K: 80.9 ± 64.0; I: 454 ± 1.19 × 10^3; H: 15.4 ± 12.5 M:	China (Lake Taihu)	LC-MS/MS	Jia et al. (2014)
Aristicthys nobilis (Bighead carp)	20.1 ± 0.56; L: 27.5 ± 24.7; K: 77.7 ± 35.0; I: 1.87 × 10^3 ± 4.32 × 10^3; H: 59.5 ± 26.7 M:			
Carassius auratus (Crucian carp)	20.1 ± 0.692; L: 19.6 ± 3.10; K: 21.9 ± 2.15; I: 24.0 ± 6.23; H: 19.0 ± 7.8			
Cyprinus carpio (Common carp)				
Cyprinus carpio (carp)	L: 221.60 ± 13.60; K: 115.00 ± 10.70; M: 5.94 ± 1.14; G: 8.05 ± 2.35; Gb: 36.31 ± 3.77; I:	India (Varanasi)	UHPLC-MS	Singh and Asthana (2014)
Clarias batrachus (Catfish group 1)	184.80 ± 12.92 L: 837.00 ± 16.65; K: 420.00 ± 12.40; M: 52.08 ± 6.52; G: 34.12 ± 3.64;			
Clarias batrachus (Catfish group 2)	Gb: 211.13 ± 9.87; I: 809.07 ± 15.60 L: 950.02 ± 18.60; 420.21 ± 11.20; M: 65.03 ± 8.71;			
	G: 38.03 ± 3.21; Sp: 3.86 ± 0.16; Gb: 227.31 ± 8.92; I: 1,005.27 ± 21.00			
Ctenopharyngodon idella (Grass Carp)	–	China (Zhejiang)	LC-MS/MS	Hu et al. (2017)
Hypophthalmichthys nobilis (Bighead carp)	L: 1.18–1.65 L: 1.00–1.62			
Mylopharyngodon piceus (Black carp)				
VEGETABLES AND CROPS				
***Plant species*/Common name**	**MC-RR concentration (ng/g)**	**Toxin source**	**Analytical method**	**References**
Lettuce	46.1 − 2352.2 (d.w.)/2.1 − 108.2 (f.w.)	Vegetable collected from the farmlands irrigated with Dianchi Lake water (China)	LC-MS/MS	Li et al. (2014a)
Water spinach	36.4 − 765.2 (d.w.)/3.1 − 65.8 (f.w.)			
Cabbage	53.1 − 394.1 (d.w.)/2.7 − 20.1 (f.w.)			
Choi sum	86.2 − 553.3 (d.w.)/6.3 − 40.4 (f.w.)			
Solanum lycopersicum	Fruits, seeds: 1.16 ± 0.67	Water from Lake Amatitlán (Guatemala)	LC-MS/MS	Romero-Oliva et al. (2014)
Capsicum annuum	Seeds: 8.10 ± 4.67			
Lactuca sativa		*Microcystis aeruginosa* crude extracts:	LC-MS/MS	Bittencourt-Oliveira et al., 2016
	ND	*1)* 0.15 μg/L MC-RR		
	6.32 ± 0.13 (d.w.)	*2)* 0.50 μg/L MC-RR		
	10.14 ± 1.34 (d.w.)	*3)* 1.50 μg/L MC-RR		
	15.00 ± 1.78 (d.w.)	*4)* 3.00 μg/L MC-RR		
Capsicum annuum	Leaves and fruits: ND	*Microcystis aeruginosa* extract 245 μg MC-LR/L	LC-MS/MS	Drobac et al. (2017)

Abbreviations: b.w.: body weight; B: brain; D: digestive gland; d.w.: dry weight; f.w.: fresh weight; G: gills; Gb: gall bladder; Gn: gonads; H: heart; I: intestine; K: kidney; L: liver/hepatopancreas; M: muscle; ND: non detected; S: stomach; Sp: spleen; V: viscera; WB: whole body. Le: leaves; F: fruits; Se: seeds; HPLC-UV: High pressure Liquid chromatography- ultraviolet detector; LC-MS/MS: Liquid chromatography— tandem mass spectrometry; UHPLC-MS: ultra-light liquid chromatography-mass spectrometry.

2018). Hu et al. (2017) reported that the quantity of MC produced and the toxin content are also related to the coexistence of various cyanobacterial strains. Hence, variant composition in cyanobacterial blooms depends on strain composition and environmental influence (Graham et al., 2010; Kaloudis et al., 2013). Moreover, Turner et al. (2018) include genetic, cellular, and population levels as decisive factors in the variability of MC congeners present in blooms. In any case, the reports reviewed in this work indicate that MC distribution in water bodies changes from surface to deep water, depending on the MC congener (Tian et al., 2013), and also from open water to a bay (Yu et al., 2014). Thus, more MC-RR than MC-LR was transported from rivers to wells (China), and current MC levels in groundwater would be considered a potential health risk to the population who drink it directly (Tian et al., 2013).

It has also been observed that several strains of cyanobacteria produce demethylated (dm-) variants of MC-RR as a sub-portion of the main toxin (Krüger et al., 2010), or, in other cases, as the main congener observed (Krüger et al., 2010; Duong et al., 2013). These dm-MC-RR congeners have been observed in water bodies not only in Asia but also in Africa (Fathalli et al., 2011; Mbukwa et al., 2012) and Europe (Faassen and Lürling, 2013). Moreover, Krüger et al. (2010) highlighted the lack of knowledge regarding the toxicity of these variants, and Mbukwa et al. (2012) remarked that the toxicity of dm-MC-RR ([D-Asp^{3}Dha^{7}MC-RR]) was higher than that of the MC-RR congener. Therefore it is necessary to take these dm-MCs into account in cyanotoxin risk assessment.

The presence of MC-producing cyanobacteria in Europe has been studied extensively. Specifically, waters containing MC-RR have been found from Portugal to Hungary (Table 1). Since its first detection several authors have reported that this variant was found as the major congener present in blooms (Rodrigues et al., 2013; Triantis et al., 2010). In this regard, Turner et al. (2018) suggested that the presence of MC-RR is associated with periods of lower rainfall. Moreover, although MC-LR has been considered the predominant MC in Portuguese waters (Martins et al., 2009; Vasconcelos, 2011), Rodrigues et al. (2013) showed comparable results for MC-RR.

Faassen and Lürling (2013) reported the presence of dm-MC-RR in Dutch waters and Ferranti et al. (2013) concluded that dm-MC-RR and MC-RR were the most abundant congeners in the Italian Lake Occhito. Similarly, dm-MC-RR was found to be the main congener produced by *Planktothrix rubescens* in Hungarian waters (Vasas et al., 2013) and, Pekar et al. (2016) reported the presence of an unspecified dm-MC-RR in Swedish lakes.

Generally, as has been shown, MC-RR is present in freshwaters. The occurrence of MCs in general and MC-RR in particular is probably higher than might be expected from the data reported in Table 1, as many countries lack monitoring programs and MC levels are not regulated. Moreover, although MC producers were usually found in freshwaters, the presence of MC producers has also been confirmed in brackish waters, probably because and MCs can reach water bodies far downstream from sites where the blooms develop (Preece et al., 2017). In this regard, Miller et al. (2010) reported that freshwater MCs are reaching the coastal areas in central California, and these MCs are causing mortality in sea otters. Therefore, the presence and distribution of MCs, particularly MC-RR, should be further investigated and monitored to avoid toxic effects on humans and wildlife.

2.2. MC-RR in food samples

MCs can bioaccumulate in various organisms used as food sources, such as bivalves, crustaceans, fish, plants, and even food supplements based on cyanobacteria species, as reviewed by Gutiérrez-Praena et al. (2013). Studies of the presence of MCs in aquatic food have been increasing in the last decade, because the global levels of MCs found could represent a risk for humans after consumption by reaching the recommended TDI value of 0.04 μg/kg body weight/day suggested by WHO (Chorus and Bartram, 1999). However, only a few works reported the concentrations found of the congener MC-RR (Table 2). This could be partly explained by the application of techniques such as ELISA, which cannot distinguish between particular congeners (Peng et al., 2010). Various reasons justify the need to distinguish the congeners implicated: 1) differences in uptake, tissue distribution, and excretion pathways may occur between different MC variants (Amé et al., 2010), and, especially, 2) there may be differences in the toxicity of MCs in terms of risk assessment.

As can be seen in Table 2, the majority of the studies dealing with MC-RR refer to aquatic food, fish being the food most frequently investigated. This is because this congener is especially taken up into tissues of aquatic biota as a result of its hydrophilic nature (Wu et al., 2013). Most of the studies report values of free MCs in aquatic matrices (Peng et al., 2010; Moreno et al., 2011; Ríos et al., 2013) because of the difficulties of extracting covalently linked MCs (Pham et al., 2017). Moreover, LC/MS and HPLC methods only detect free toxins (Moreno et al., 2005), and MC covalently bonded to proteins would not react with ELISA because the toxin is sterically blocked by the protein (Peng et al., 2010). However, in terms of risk assessment the covalently bonded portion of MC-RR (or total MCs) is not detectable by conventional organic solvent extraction methods and may represent up to 38–99% of the total MC contained in food. This may lead to an underestimate of the human health risks (De Pace et al., 2014; Catherine et al., 2017), because the bonded MC could be released during digestion processes. Recently, Pham et al. (2017) reported an average of covalently bonded MC of 19.3% in a small number of fish and bivalve samples.

The food source with the highest MC-RR content are bivalves, followed by fish. This is reasonable, given that bivalves are filtering organisms and fish are known to graze and feed on cyanobacteria species. It is noteworthy that most of the studies have been conducted in Asia, particularly China, where it is well known that contamination with cyanobacteria is an endemic problem. In bivalves, the detection of MC in wild and farmed mussels raises the problem of possible transference through the food to marine predators, although studies are very scarce. De Pace et al. (2014) identified and reported for the first time high concentrations of the MC variant dm-MC-RR describing the direct relationship between the temporal trend of bloom presence in lake surface waters and toxin contamination in mussels. The accumulation of MC-RR in three freshwater bivalves has been demonstrated in muscle and gland tissues of mussels in in situ ponds, and differences in bioaccumulation patterns among these filter feeders could be due to interspecific differences in selective ingestion and depuration rates (Kim et al., 2017).

The presence of MC-RR has been reported in snails (*Bellamy*a genus), mainly in bile and digestive tracts, but it does not represent risks for human health because these parts are removed prior to consumption (Piontek and Czyzewska, 2017).

In crustaceans, only two studies demonstrate the bioaccumulation of MC-RR in the crayfish *P. clarkii* in ponds in Spain (Ríos et al., 2013) and China (Chen and Xie, 2005), confirming the presence of MCs in this and other crayfish species reported in earlier studies (Vasconcelos et al., 2011; Chen and Xie, 2005). Ríos et al. (2013) reported that MC-LR was the predominant toxin detected in crayfish and indicated that fish living in the contaminated ponds were not able to accumulate detectable quantities of MC. Moreover, Chen and Xie (2005) observed that the concentration of MC-RR in *P. clarkii* decreased in the following order: stomach > intestines > gonad > gills > muscle. The presence of MC-RR in the crustaceans *Macrobrachium nipponense* and *Eriocheir sinensis* H. Milne-Edwards was demonstrated by Peng et al. (2010), the estimated daily intake (EDI) values for MCs being higher than the TDI value, so these crustaceans seem to be unsafe for human consumption.

In fish, several studies performed under natural conditions demonstrate the accumulation of MC-RR in various tissues via the blood stream, mostly in liver and kidney (Table 2), and this accumulation

could lead to human exposure through the diet. Peng et al. (2010) evaluated the EDI of MCs (including MC-RR) in 13 fish species and reported that all of them were deemed unsafe for consumption because EDI values were 5–148 times higher than the TDI value.

Xie et al. (2005) reported the presence of MC-RR in phytoplanktivorous, omnivorous, and carnivorous species of fish collected from Lake Chaou in China. Moreover, these authors indicate other uptake routes of MCs, besides via gastrointestinal tract, owing to the presence of MC-RR in muscle of carnivorous fish despite the absence of MCs in their gut.

Although most of the studies have been carried out in Asia, MC-RR was the dominant variant in water from lakes in Argentina, and in liver and muscle of the fish (*O. bonariensis*) collected (Amé et al., 2010), reaching concentrations in the liver 10-fold higher than in fish muscle. A significant correlation between MC-RR concentrations in the two organs was found. It was also noteworthy that MC-RR was found in fish muscle during the dry season, even though it was not present in water samples during the same period, and according to these authors this could be useful to indicate the temporal variability of bloom toxicity (Amé et al., 2010). It was hypothesized that metabolic depuration is more effective with some MC variants than with others, leading to higher accumulation of those variants that cannot be easily eliminated from fish tissues (Deblois et al., 2008). The dominance of MC-RR in fish tissue can be explained in various ways: a) the presence of this congener in the water column, and b) fast uptake and slow depuration of MC-RR compared to other MC variants (Singh and Asthana, 2014). Moreover, environmental factors (temperature, etc.) could also affect accumulation of MC variants in fish tissues, and further studies on this aspect are recommended by various authors to ascertain the toxicokinetic differences of MCs in fish and aquatic food.

In natural conditions, carp cultured in contaminated lakes, such as Lake Taihu, showed high bioaccumulation of MC-RR, especially in liver and kidney, followed by muscle, and there was a correlation between MC-RR contents in liver and kidney (Wu et al., 2013). A liver/kidney ratio of 2 indicated that the kidney was active in depuration of MC-RR, showing less accumulation of the toxin. In silver carp from various eutrophic lakes in China, MC-RR was the most prominent MC congener in fish muscle, in agreement with other previous studies carried out in several species of fish in Lake Taihu (Zhang et al., 2009), or in Nile tilapias in Lake Mburo (Uganda), accounting for > 70% of detected MC in the latter location (Semyalo et al., 2010). The length of silver carp in these conditions showed a negative correlation with MC concentrations (MC-RR being predominant) in their muscle (Zhang et al., 2013). These authors suggested that MCs accumulation in silver carp muscle seems to be size dependent, maybe because different total length of this species has different detoxification and/or excretion efficiency of these toxins. In contrast, no correlations between environmental variables and MC-RR concentration were found in Nile tilapia, whose levels of MC-RR (and total MCs) were highest in the gut, followed by liver and then muscle tissue, indicating that the contents found were significantly lower than the amount of toxins ingested (Semyalo et al., 2010).

Jia et al. (2014) examined the accumulation of specific MCs, such as MC-RR, in four species of fish from various trophic levels and feeding guilds. In phytoplanktivorous fish species the heart had concentrations of MC-RR that accounted for 100% of total MCs, followed by the intestinal wall (76–96%), muscle (70–75%), kidney (67–87%) and liver (58–66%). The MC-RR concentrations were significantly higher in the intestinal wall and decreased in the following order: kidney > liver > muscle > heart. By contrast, the two omnivorous fish species studied exhibited differential accumulation of MC-RR (and other MCs) in the organs analyzed, although this congener accounted for the largest percentage of the total MCs (100%) in the heart. No significant differences were observed in MC-RR concentration between phytoplanktivorous and omnivorous fish, suggesting that bioaccumulation of MC depends on the species feeding guild, duration of exposure to the toxin, rates of accumulation relative to depuration, and metabolic rates of the fish species. The accumulation of MC-RR (and other MC congeners) in the intestinal wall in comparison to other organs could inhibit further transport of MCs into internal organs. The MC-RR concentrations in the muscle were not significantly correlated with the values found in the liver, kidney, intestinal wall, or heart. The authors suggested that consumption of algal cells was the main factor that influenced the accumulation of MC-RR (and MCs in general) by fish. This could explain the differences found in the distribution of MC-RR in organs of fish from the same study site (Lake Taihu, China) (Wu et al., 2013).

In another geographical location (a lake in India), the MC-RR concentrations in omnivorous fish followed the order: liver > gut > kidney > gall bladder > gills > muscles for carp, and gut > liver > kidney > gall bladder > muscles > gills for catfish (Singh and Asthana, 2014). The higher MC-RR content in organs other than the liver (target organ) in catfish suggested the capacity of these organs to bioaccumulate this congener (Magalhaes et al., 2001; Xie et al., 2005), because the major route of fish exposure to MCs is through oral ingestion of water containing dissolved MCs. Moreover, bioaccumulation of MC-RR was species-specific in the fish, and the lower levels of MC-RR in carp compared to catfish could reflect the decreased grazing rate of carp in the presence of toxic cyanobacteria. According to Singh and Asthana (2014), even within the same species of fish, catfish, with higher body weight and size, accumulated lower MC-RR levels and MC-LR levels, reflecting a negative relationship between bioaccumulation of MC and body weight and fish size, maybe due to physiological differences (e.g. water pumping, respiration, etc.). Previous works indicated that bioaccumulation of MCs in fish varied widely within individuals of the same species (Vasconcelos, 1999). Recently, Pham et al. (2017) reported that in two omnivorous fish (*B. gonionotus* and *P. proctozystron*) the liver had the greatest proportion of MC-RR (80% total MCs measured), followed by intestines, in agreement with the sequence found by Singh and Asthana (2014), whereas the highest proportion of MC-RR in the phytoplanktivorous fish (*O. niloticus*) was in intestines.

In recent years, experiments under laboratory conditions for MC-RR were very scarce. Xie et al. (2004) found concentrations of 49.7, 17.8, and 1.77 μg/g of MC-RR in blood, liver, and muscle of silver carp exposed to MC through food uptake. In carp injected intraperitoneally (i.p.) with extracted MC, not only was MC-RR detected in the liver of the fish, but metabolites of it, such as MC-RR-GSH and MC-RR-Cys, were also detected (Wu et al., 2010). In crucian carp, the kidney showed a dose time-dependent increase in MC-RR content within 48 h post-injection followed by a sharp decline, while no MC-LR was detectable (Li et al., 2013). These differences might be due to organ specificity of different MC-congeners, MC-RR being accumulated in kidney rather than MC-LR, and mainly promoting ultrastructural pathological changes in renal membranous structure and causing renal dysfunction. This may be related to its higher hydrophilicity in comparison to MC-LR (Prieto et al., 2006).

Bioaccumulation of MC in plants used for feed and food has been reported in various species (lettuce, broccoli and mustard roots, rice and rape seedlings, etc.) whose levels of MC, without specifying the MC congener, were reviewed by Gutiérrez-Praena et al. (2013), including their consequences on animals and human health (Corbel et al., 2014). Under laboratory conditions, bioaccumulation of MC in edible plants, such as foliar tissues of lettuce (*Lactuca sativa* L.) sprayed with water containing specific congeners (MC-LR, MC-RR) at environmentally significant concentrations, demonstrated that a longer exposure time resulted in greater bioaccumulation, reaching MC levels higher than the TDI recommended by WHO (Hereman and Bittencourt-Oliveira, 2012). The accumulation depends on various factors, including the toxin variant (Saqrane et al., 2009; Romero-Oliva et al., 2014), dose, duration of exposure, modes of exposure, and plant organ (Drobac et al., 2017), and the risks for human health are plant-specific for leafy vegetables (Crush et al., 2008).

As in the case of aquatic food, in most of the studies of MC bioaccumulation in vegetables the determination of toxin content was carried out using radiotracer, or ELISA (Crush et al., 2008) and protein

phosphatase inhibition assays (PPIA) (Järvenpää et al., 2007), and consequently no values of individual MC-congeners, such as MC-RR, were reported. Very few authors have studied the simultaneous presence of MC variants, particularly MC-RR, in vegetable matrices (Table 2). Li et al. (2014a) determined several MC congeners in 28 field vegetable samples, and MC-RR was the most frequently detected MC in almost all samples (89.3%).

A detailed study of lettuce irrigated with different concentrations of MC-RR (0.15–3.0 μg/L) for 15 days found a significant positive correlation between the amounts of MC-RR (and total MCs) detected and the various exposure concentrations (Bittencourt-Oliveira et al., 2016). No detectable levels of MC-RR were found at the lowest exposure concentration and the bioaccumulation factor (BAF) for MC-RR varied according to the exposure concentrations. Changes in BAF for the various MCs observed in lettuce were not directly proportional to MC exposure concentration, so higher concentrations might not always result in higher BAFs, as explained by Crush et al. (2008).

Edible plants not only accumulated MCs on their surface (leaves), but also in their tissues and organs, including roots and shoots. Romero-Oliva et al. (2014) evaluated the presence of MCs in *S. lycopersicum* and *C. annuum* and MC-RR contributed to more than 80% of the total MC concentration. Translocation of MC-RR may explain its presence in fruits and even in new crop plants via their seeds. The values obtained were below the TDI recommended, but the authors indicated that they could have been affected by the sampling period because the plants were irrigated during the rainy season, with potentially lower availability of MCs. In contrast, in leaves and fruits of *C. annuum* irrigated with diluted extracts of MCs (containing several MC congeners) applied to the soil for a period of 3 months, MC-RR and dm-MC-RR did not accumulate. Only the variants MC-LR and dm-MC-LR were detected in fruits (Drobac et al., 2017). There could be various explanations for this lower bioaccumulation of MCs resulting from the application of contaminated water to soil: 1) the action of microorganisms that are present in soil and capable of degrading cyanotoxins (Li and Pan, 2015), or 2) adsorption of MCs in soil might reduce the concentration of free MC-RR in the aqueous phase in contact with plants (Järvenpää et al., 2007). These authors investigated uptake of MCs by vegetables in a realistic scenario, where the plants were grown in soil/vermiculite. MC elimination from the water phase by soil and vermiculite reduced the concentration of toxin available to the plants and explained the low MC concentration observed, and even MC-RR was not detected. The more polar MC-RR (net charge 0 at neutral pH) was more quickly adsorbed by soil, vermiculite or a mixture of them than the medium-polar MC-LR (net charge –1 at neutral pH).

Broadly speaking, it is clear that MC-RR occurrence data are more numerous in water than in food sources, but in view of the close link between them further reports dealing with MC-contaminated food products and their wider geographical distribution can be expected. Therefore, regular monitoring of MC-RR and MC in general in water and food are recommended in terms of risk assessment, as well as further studies on this topic.

3. Toxicokinetics

It has been pointed out that the toxicodynamics of the various congeners of MC do not play an important role in the toxicity of these cyanotoxins (Fischer et al., 2010; Buratti et al., 2017). It seems that the toxicokinetics of these toxins is one of the factors that contributes to their toxicity (Steiner et al., 2016). Absorption, distribution, biotransformation, and excretion may be affected by modifications in the chemical structure of the molecule (Dietrich and Hoeger, 2005; Buratti et al., 2013), which can result in threefold to fourfold differences among some congeners (Meriluoto et al., 1990).

3.1. Absorption

With regard to absorption, as a result of their structure and amino acid composition MCs are rather hydrophilic and spatially large molecules. Therefore they are apparently incapable of crossing cell membranes via passive diffusion, and instead require active transport via specific transporters (Fischer et al., 2010). It is well established that organic anion transporter polypeptides (OATPs) are responsible for MC transport into cells (Ibelings and Chorus, 2007). Their involvement in MC uptake has been demonstrated in several experimental models, including fish, mice, rats, and humans (Drobac et al., 2017). Moreover, there could be differences between the affinities of MC variants to OATPs (Ibelings and Chorus, 2007). Also, as MC-RR is more polar than MC-LR, it is not as easily transported via the bile acid transport system as MC-LR (Schmidt et al., 2014).

Cazenave et al. (2005) demonstrated that MC-RR could be absorbed through the gastrointestinal tract (g.i.) and gills, because they found different amounts of this toxin in these organs after 24 h of exposure. However, they remarked that the absorption mechanism associated with gills was not very clear, although Drobac et al. (2017) still consider this route as plausible.

3.2. Distribution

Once MC-RR is absorbed, it is distributed through the blood stream to the organs, mainly well-irrigated organs such as liver, kidney, intestine, lung, brain, etc. (Wang et al., 2008). However, as Schmidt et al. (2014) pointed out, this distribution is subject to various factors that could affect the process, such as the animal model, administration method, MC congener, etc. With regard to MC-RR, Xie et al. (2004) studied its levels in blood of silver carp, finding concentrations that were very low or below their limit of detection (LOD) after 20 days of subchronic exposure, assuming that MC-RR rapidly reaches tissues. These results were corroborated in bighead carp by Li et al. (2007a).

The expression of different OATP subtypes together with selectiveness and ability to transport MC congeners could play an important role in the toxicity of these cyanotoxins (Steiner et al., 2016). The uptake of MCs through OATPs has been described as congener-dependent, because several authors have found that there is selective uptake of MCs in cells, depending on the organ (Fischer et al., 2010; Zeller et al., 2011). Thus the organotropism associated with MC could be due to the high OATP expression that occurs in hepatocytes (Cazenave et al., 2005; Zeller et al., 2011). In this regard, the latter authors demonstrated that the presence of specific OATPs, 4A1 and 3A1, in Caco-2 cell membrane facilitated entry of MC-RR into them. Moreover, Fischer et al. (2010) found that OATPs do not have the same affinity for all MC congeners, showing that hepatic OATPs 1B1 and 1B3 facilitated entry of MC-LR into the hepatocyte while MC-RR impeded entry. Therefore, MC-RR could accumulate in tissues (Drobac et al., 2017). Cazenave et al. (2005) demonstrated the accumulation of MC-RR in liver, intestine, muscle, and brain of three different catfish species, the liver being the organ with the highest levels of MC-RR and the brain the one with the lowest level. This is in agreement with results obtained by Li et al. (2005a, 2007a), who found that liver accumulated great amounts of MC-RR, although they were rapidly cleared after 48 h. Zhang et al. (2007) also found that MC-RR accumulated mainly in intestine, liver, and muscles of silver carp, whereas its presence in kidney was minimal. By contrast, Li et al. (2007a) found that MC-RR accumulated mainly in kidney of bighead carp acutely exposed to this toxin, followed by gallbladder, intestine, muscle, and spleen.

In Wistar rats, Wang et al. (2008) found MC-RR in several organs, detecting the highest contents in gonads, liver, heart, stomach, and kidney. Generally, the accumulation of MC-RR in organs is OATP-mediated (Drobac et al., 2017). More specifically, the fact that the liver is the main organ that accumulates MC-RR could be explained by the amount of OATPs present in this organ. It could also be linked to low

biotransformation rates, that could lead to accumulation of the toxin in the organ, although this usually happens when high concentrations of MC-RR are administered (Cazenave et al., 2005). In the case of intestine, as these authors also stated, the presence of MC-RR in this tissue could be derived from ingestion of the toxin through contaminated water, but it could also be due to enterohepatic recirculation and desorption from the blood stream. The accumulation of MC-RR in the brain has been explained by the presence of OATPs in the blood–brain barrier, which facilitate entry of the toxin into the central nervous system (Cazenave et al., 2005; Fischer et al., 2005). For muscles, the main theory for MC-RR accumulation is that the toxin enters through the system of arterioles, venules, and capillaries, as a similar system of OATPs has not been found in muscles (Drobac et al., 2017).

In plants, Yin et al. (2005a) demonstrated that MC-RR accumulates in roots and leaves of the aquatic macrophyte *Vallisneria natans* in a concentration-dependent manner.

3.3. Metabolism

With regard to the biotransformation of MC-RR, it is well established that it happens through conjugation with GSH in mammals, fish, and plants (Buratti et al., 2013; Buratti and Testai, 2015). This conjugation leads to less toxic MC derivatives, so the rate of biotransformation also plays a role in the toxicity of MC-RR (Kondo et al., 1992; Buratti et al., 2013; Buratti and Testai, 2015). Moreover, it has also been demonstrated that some MC congeners can be more efficiently metabolized than others (Buratti et al., 2013). The conjugates are rapidly formed at alkaline pH and enzymatically catalyzed by glutathione-S-transferase (GST) (Pavagadhi et al., 2012; Buratti et al., 2013; Schmidt et al., 2014; Buratti and Testai, 2015; Pflugmacher, 2016). In this regard, Cazenave et al. (2006a) found that MC-RR is able to inhibit GST in catfish, so the metabolization rate could be affected, with the biologically derived consequences.

The first step of the conjugation occurs through the Mdha residue of MC and the cysteine group of GSH (Schmidt et al., 2014). A detailed explanation of MC conjugation with GSH can be found in Martins and Vasconcelos (2009). In their review the authors indicate that conjugation may significantly reduce the rate of PP inhibition by MC-RR, but also that a high rate of conjugation could lead to an oxidative stress situation. This latter situation has also been corroborated by Pavagadhi et al. (2012) in zebrafish.

Buratti et al. (2013) and Buratti and Testai (2015) found a similar trend in rodents and humans, although they observed some differences between them. For example, they found that rodents showed a more marked metabolism of MC-RR than humans, probably owing to the different GST isoforms expressed by the two organisms, and this could be due to the extensive polymorphism of this enzyme in humans, even presenting a complete lack of activity. However, when the MC-RR concentration is low the enzymatic reaction increases, reaching high levels of detoxication (Buratti et al., 2013). Nevertheless, when GSTs were studied, it seemed that GST isoforms GSTT1-1, GSTA1-1, and GSTM1-1 presented the highest affinity and catalytic activity against MC-RR (Buratti et al., 2013). Once the MC-RR-GSH conjugate is formed, it is rapidly converted into MC-RR-Cys, as demonstrated by Li et al. (2014b, 2018), who found high levels of this conjugate instead of MC-RR-GSH conjugates, mainly in kidney of bighead carp and rats, although it was also found in intestine. Finally, MC-RR-Cys conjugates are transformed into MC-RR-N-acetylcysteine (Nac) conjugates, which are ready to be excreted. However, the same authors also found that MC-RR conjugates may dissociate, with the toxin appearing alone and increasing its toxicity, not only in the liver but also in other organs (Li et al., 2014b, 2018). Moreover, Schmidt et al. (2014) pointed out that the enterohepatic cycle and metabolism could also induce the appearance of other different MC analogues with different unknown toxicity profiles. Wu et al. (2013) also observed that the concentration of MC-RR-Cys in kidney was 4 times greater than in liver of common carp. Moreover, ratios of MC-RR-Cys/MC-RR in liver (5.3)/kidney (39.8) indicated that kidney showed low accumulation of MC-RR and higher formation efficiency of MC-RR-Cys (7.5 times faster) than liver, suggesting that MC-RR-Cys was significantly accumulated with the depletion of MC-RR, and MC-RR was selectively biotransformed to MC-RR-Cys in kidney for subsequent elimination. The high ratio of MC-RR-Cys/MC-RR was good evidence that common carp formed MC-RR-Cys efficiently in a long-term bloom, suggesting that cysteine S-conjugates were important metabolites of MC-RR and that kidney showed an active mechanism for depuration of MC-RR by formation and accumulation of MC-RR-Cys.

In plants, Huang et al. (2008a) demonstrated in BY-2 cells that MC-RR was biotransformed via GSH conjugation.

3.4. Excretion

Excretion of MC-RR in mammals has been found to be as MC-RR-GSH and MC-RR-Cys conjugates, but also as MC-RR in urine, the MC-RR-Cys conjugate being the main form present in the urine. In feces, these compounds were also found, but in lower quantities, although MC-RR was predominant over the other forms (Li et al., 2018). These authors demonstrated for the first time that parent MC-RR excretion occurs mainly by feces in mammals, which was in agreement with results published by Li et al. (2014c) for fish, demonstrating that the reversible ability of MC conjugates is an inherent characteristic in them. The excretion of MC-RR through feces implies that MC-RR is transported faster to the intestine, which is also related to minimal changes in its structure when it is conjugated, changing the affinity for OATP transporters, or is also due to rapid excretion of this compound in bile salts (Li et al., 2014b, 2014c).

In general, the toxicokinetics of MC-RR is now better known because many studies have focused on this issue recently, as shown above. Thus, its uptake and transport is mediated by OATPs and its elimination is through conjugation with GSH appearing mainly in urine but also in feces, as MC-RR or its metabolites. However, there are still some gaps to be covered. In this regard, information about its uptake mechanisms is still scarce, and more research should be done on the specific affinity of OATPs for MC-RR or other transport mechanisms. The possibility of acting to inhibit the uptake of this toxin in cases of exposure could prevent its toxic effects. Similarly, studies on elimination mechanisms should be carried out to facilitate complete clearance of MC-RR once it has entered the organism.

4. Toxicity

4.1. General aspects

Toxicity mechanisms of MCs have been widely investigated. It is well established that MCs inhibit protein serine/threonine phosphatases, especially PP1 and PP2A by covalent binding (Mackintosh et al., 1990), but also PP3–PP6, whereas no inhibition of PP2B, PP2C, and PP7 was observed (Fischer et al., 2010). This inhibition leads to protein hyperphosphorylation, reversion of the action of protein kinases, and, as a consequence, modification of cytoskeleton and disruption of actin filaments (Buratti et al., 2017). MCs can also induce oxidative stress by increasing the formation of reactive oxygen species (ROS) and/or by GSH depletion (Campos and Vasconcelos, 2010) leading to lipid peroxidation (LPO) and alteration of antioxidant enzyme activities (Valério et al., 2016). Oxidative stress can also activate the c-Jun N-terminal protein kinase (JNK) (Shen and Liu, 2006; Kaneto et al., 2005) and its downstream targets, AP-1 and Bid. This can influence crucial enzymes of energy metabolism, leading to mitochondria dysfunction, which contributes to the intrinsic apoptosis pathway (Wei et al., 2008; Chen and Xie, 2016). Reduced DNA repair and cell proliferation leading to tumor promotion are also toxicity mechanisms associated with MCs (Buratti et al., 2017). In this regard, MC-LR was classified as “possibly

Table 3
In vitro cytotoxicity studies performed with MC-RR.

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
Commercial pure MC-RR standard (and MC-LR)	RTG-2 and PLHC-1 cells	Protein content Neutral Red LDH leakage MTS reduction Morphological changes	0, 50, 100 μM for 24 h	PC was the most sensitive endpoint for both cell lines with an EC_{50} of 79 μM for PLHC-1 and 195 μM for RTG-2. Only PLHC-1 cells were sensitive to LDH leakage. Thus, MC-RR was much more toxic to PLHC-1 than to RTG-2 cells. 50 μM MC-RR produced a clear microvesicular steatosis in PLHC-1 cells and 100 μM in RTG-2 cells produced a clear hydropic degeneration of cytoplasm.	Pichardo et al. (2005)
MC-RR from a purified extract of *Microcystis aeruginosa*	Tobacco BY-2 cells	Cell viability by Evan´s blue exclusion	0 and 50 mg/L for 12, 24, 48, 72, 96, 120 and 144 h. Later 168 h recovery	Significant cytotoxic effects with respect to the control were observed in the cells between 48 and 144 h of exposure. After the recovery period the viability increased from 80% to 89%.	Yin et al. (2005a, 2005b, 2005c)
MC-RR from a purified extract of *M. aeruginosa*	Tobacco BY − 2 cells	Apoptosis by flow cytometry and microscopy	0, 0.1Javurek, 1, 10, 50 mg/L for 0, 2, 4, 6 and 8 days	Apoptosis was observed at the higher concentration assessed (50 mg/L) after 6 days of exposure by microscopy and after 8 days by flow cytometry. Apoptotic cells were observed in a time and concentration-dependent manner.	Yin et al. (2006)
Commercial pure MC-RR standard (and MC-LR)	Lymphocytes of crucian carp *(Carassius auratus)*	Apoptosis by confocal microscopy and TEM, gel electrophoresis and flow cytometry	1 nM for 2 h: confocal microscopy 1 nM for 4 h: TEM 1, 5 and 10 nM for 2 h: gel electrophoresis 1 nM for 2, 4, 6 and 8 h: flow cytometry	Condensation of nucleus and formation of apoptotic bodies were observed among other morphological changes. A DNA ladder pattern was evidenced. MC-RR induced apoptotic cells in a time and concentration dependent-manner by flow cytometry.	Zhang et al. (2006)
Commercial pure MC-RR standard (also MC-LR, -YR, -LF, -LW)	OATP- transfected HeLa cells	Sulforhodamine B protein dye assay	Concentrations not stated	Cytotoxic effects were observed in OATP-transfected cells. IC_{50} values were 3.8 μM and 0.58 μM, OATP1B1 and OATP1B3 transfected cells, respectively.	Monks et al. (2007)
Purified extract from *M. aeruginosa*	Tobacco BY − 2 cells	TTC reduction assay	0.1, 1, 10 μg/mL for 0, 3, 4 and 6 days	Cell viability decreased in a time- and concentration-dependent manner. After 4 and 6 days the toxin-treated groups showed significant differences respect to the control with 1 and 10 μg/mL MC-RR.	Huang et al. (2008a)
Purified extract containing MC-RR	Tobacco BY − 2 cells	Morphological changes Apoptosis by flow cytometry	Morphology: 60 μg/mL for 5 days Flow cytometry: 60 μg/mL for 1, 2, 3, 4 and 5 d	A significant decrease of the cellular width was observed as well as fragmentation of nucleus and numerous apoptotic nuclei. MC-RR induced rapid apoptosis in a time- dependent manner.	Huang et al. (2008b)
Purified extract containing MC-RR	Tobacco BY − 2 cells	Evan´s blue exclusion test Morphological changes	60 and 120 μg/mL for 3 and 5 days	After exposure, the cells underwent different morphological changes typical of apoptosis and necrosis according to the exposure concentration. Exposure to 60 μg/mL of MC-RR induced apoptotic cells while necrotic cells were observed when cells were exposed to 120 μg/mL of MC-RR.	Huang et al. (2009)
Commercial pure MC-RR standard (and MC-YR)	Undifferentiated and differentiated Caco − 2 cells	PC, NRU and MTS assays Morphological changes	50, 100, 150, 200 μM for 24 and 48 h	The highest cytotoxic effects were observed in PC after 24 and 48 h in undifferentiated and differentiated cells, being significant from 100 μM at both times of exposure. In differentiated cells, a reduction of 45% in PC respect to the control was observed after 48 h to 200 μM MC-RR. General reduction in cell number and hydropic degeneration was observed at high concentrations.	Puerto et al. (2009)

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Table 3 (*continued*)

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
Purified extract containing MC-RR (among others)	Human normal hepatocytes	MTS assay	0–100 nM for 3 days	Cytotoxic effects were observed. A weak relationship between cytotocixity and PP2A assay was reported. The IC_{50} and LC_{50} values were 0.056 nM and 158 nM for the PP2A inhibition assays and MTS, respectively.	Ikehara et al. (2009)
Commercial pure MC-RR standard (also MC-LR, -LF, LW)	Primary human hepatocytes and OATP- transfected HEK293 cells	MTT assay	2.29–5000 nM for 48 Cytotoxic effects were observedh	OATP- transfected HEK293 cells were resistant to the toxicity induced by MC-RR. Primary human hepatocytes showed major sensitivity to MC-RR. The EC_{50} values obtained in primary human hepatocytes were different between donors. The EC_{50} at donor 1 was 167.2 nM while in donor 2 was 900.2 nM. In OATP-transfected HEK293 cells the EC_{50} were 1267 nM in HEK293-OATP1B3 cells and $>$ 5000 nM in HEK293-OATP1B1 cells.	Fischer et al. (2010)
Commercial pure MC-RR standard (and MC-LR)	Caco − 2 cells	MTT assay	1–50 μM for 30 min to 24 h	No cytotoxicity was observed in Caco − 2 cells after 24 h of exposure.	Zeller et al. (2011)
Commercial pure MC-RR standard (and MC-LR) and purified extracts from *M. aeruginosa, M. wesenbergii, M. ichthyoblabe* and *Aphanizomenon* sp.	HaCat and H4IIE.luc cells	NRU and MTT assays	6–100 mg/L for single MC, 0.0078–1 g dw/L for extracts of laboratory single cyanobacterial species and 7.8×10^{-5}–2 g dw/L for samples from environmental biomasses for 72 h	After 72 h of exposure, the MTT assay showed cytotoxic effects with an EC_{50} value of 45 mg/L MC-RR for H4IIE.luc and 57 mg/L MC-RR for HaCat cells. NRU assay was less sensitive. Less cytotoxic effects were observed when these cell lines were exposed to extracts of cyanobacterial blooms.	Štěpánková et al. (2011)
Pure MC-RR (and MC-LR, YR and NOD) and/or purified from *M. aeruginosa* extract	Primary human and rat hepatocytes	Resazurin reduction assay and LDH leakage	0–100 nM for 24 h.	MC-RR was not cytotoxic in human and rat hepatocytes after 24 h of exposure.	Ufelmann et al. (2012)
Commercial pure MC-RR standard (and MC-LR)	Differentiated Caco − 2 cells	XTT reduction, NRU and PC assays	1, 5, 10, 50, 80, 100 μM for 4 and 24 h	No significant cytotoxicity was observed for Caco − 2 cells exposed to MC-RR for 4 h. Significant cytotoxic effects were observed in PC and NRU assays after 24 h of treatment. At the two higher concentrations (80 and 100 μM), cytotoxic effects were observed achieving only a maximum of 25%.	Huguet et al. (2013)
MC-RR and other 15 variants from purified extracts from *M. aeruginosa, Planktothrix agardhii* or *Planktothrix rubescens*	Primary cultured rat hepatocytes	Cellular ATP content	0–10 μg/mL for 72 h	Cytotoxicity results were different between MC-RR and its congeners ([D-Asp3, Dha7] MC-RR, [D-Asp3, E-Dhb7] MC-RR, [Dha7] MC-RR and [D-Asp3] MC-RR). MC-RR was the less toxic of all the substances assessed with an IC_{50} $>$ 10 μg/mL while its variants showed greater cytotoxicity.	Shimizu et al. (2013)
MC-RR from a purified extract of *M. aeruginosa*	HeLa cells	CCK8 kit Cell cycle and apoptosis by flow cytometry Gene and protein expression	0–80 μg/mL for 4, 8, 12, and 24 h	MC-RR significantly increased cell proliferation with the increment of concentration, except at the highest concentration assayed (80 μg/mL), which showed no significant differences with respect to the control group. In the cell cycle/ apoptosis study, low concentrations promoted G1/S transition and cell proliferation through the expression of NF-kB, while the higher ones induced apoptosis.	Chen et al. (2014)
MC-RR extracted and purified	Tobacco BY − 2 cells	Apoptosis by flow cytometry Morphological changes	60 mg/L for 3 and 6 days	A significant increase in apoptotic cells after exposure to 60 mg/L MC-RR was observed. Condensation and fragmentation of nuclei, characteristic of apoptotic cells.	Huang et al. (2014)
Purified extract from *M. aeruginosa*	P19/A15 cells	NRU assay	0.25, 0.5, 1, 2 g dm/L for 24 h	No cytotoxicity effects were described.	Javůrek et al., 2015

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Table 3 (*continued*)

Toxin	Experimental model	Assays Performed	Concentration ranges and exposure conditions	Main results	References
Commercial pure MC-RR standard (and MC-LR, -LY)	HepG2 cells	MTS assay	0.1 − 10 μM for 24 h	MC-RR was non-cytotoxic to HepG2 cells after 24 h of exposure showing cell viability greater to 80% respect to the control.	Lundqvist et al. (2017)

Abbreviations: Caco-2: Human colorectal adenocarcinoma cell line; CCK8: WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; HaCat: Human keratinocytes immortalized skin cell line; H4IIE.luc: *Rattus norvegicus* liver hepatoma cell line; HEK293: Human embryonic kidney cell line; HeLa: Human cervix epitheloid carcinoma cell line; Hep-G2: Human liver hepatocellular carcinoma cell line; LDH: Lactate Dehydrogenase; MTS assay: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); MTT assay: 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NOD: Nodularin; NRU: Neutral red uptake; OATP: Organic anion transporting polypeptide; P19/A15: Murine embryonic carcinoma cells derived from P19 cell line; PC: Protein content; PLHC-1: *Poeciliopsis lucida* hepatocellular carcinoma cell line; Resazurin: 7-Hydroxy- 3H-phenoxazin-3-one 10-oxide; RTG-2: *Oncorhynchus mykiss* trout Rainbow fibroblast gonad tissue cell line; TEM: transmission electron microscopy; Tobacco BY-2: *Nicotiana tabacum* L. cv. Bright Yellow 2 cell line; TTC: (2, 3, 5-triphenyl-tetrazolium chloride); XTT: sodium 2,3,-bis(2 methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium.

carcinogenic to humans" (Group 2B) by the International Agency for Research on Cancer (IARC). Other MC congeners have not been evaluated so far.

No specific toxicity mechanisms for MC-RR have been reported other than those already known for MCs in general. MC-RR causes PP inhibition (Robillot and Hennion, 2004), oxidative stress (Cazenave et al., 2006a, 2006b; Huang et al., 2008b), etc. However, the toxic potency among MC variants changes remarkably. The in vivo acute toxicity is 10-fold lower for MC-RR in comparison to MC-LR (i.p. LD_{50} in mice: 500 and 50 μg/kg, respectively) (Funari and Testai, 2008). A compilation of acute toxicity data (LD_{50}) for MC-RR can be found in Buratti et al. (2017), with values between 111 and 650 μg/kg b.w. in mouse by i.p. injection. The individual kinetics profile appeared to be the main reason explaining the differences observed (Buratti et al., 2013), as mentioned earlier (Section 3). Moreover, Hoeger et al. (2007) suggested that the PP-inhibiting capacity and the ability to form covalent bonds to enzymes of the PP family (this is toxicodynamics) do not consistently explain the in vivo toxicity observed. This suggestion was made on the basis of the similar IC_{50} results obtained for PP1 and PP2A by MC-LR and MC-RR, with values of 2.5 (1.7–3.7) and 1.9 (1.3–3.0) nM respectively for PP1 and 0.68 (0.64–0.74) and 0.75 (0.59–0.96) nM for PP2A. Other authors also agreed regarding similar IC_{50} values in the nanomolar range for various MC congeners (Robillot and Hennion, 2004; Monks et al., 2007), suggesting a highly conserved molecular mode of action (Fischer et al., 2010). Moreover, the demethylated variant [Asp^3]MC-RR showed a similar covalent binding potential to MC-RR, but in contrast the demethylated variant [Asp^3, Dhb^7]MC-RR (with dehydrobutyric acid at position 7) did not covalently bind to PP1 by western blot (Hoeger et al., 2007). These authors suggested that the combination of two arginines at positions 2 and 4 with Dhb at position 7 may be sterically hindering and thus restrict binding of [Asp^3, Dhb^7]MC-RR to the catalytic subunit of PP. However, despite the far lower PP-inhibiting capacity of this variant, it has in vivo LD_{50} values comparable to those of MC-RR, so the PP1A analysis of [Asp^3, Dhb^7]MC-RR may lead to underestimation of potential toxicity. Moreover, these data suggest that, although MC-RR and MC-LR do not differ in their PP-inhibiting capacity, other structural changes in the molecule may do so, highlighting the importance of structure not only in toxicokinetics but also in toxicodynamics.

Also, apart from the specific MC congener toxicity observed in vivo, distinct differences between MC-LR and -RR toxicity have also been reported in in vitro studies. Thus, Fastner et al. (1995) and Eriksson et al. (1990) demonstrated that primary rat hepatocytes were less susceptible to MC-RR (EC_{50} 1500–4300 nM) than to MC-LR (EC_{50} 60–200 nM). Moreover Fischer et al. (2010) observed that, irrespective of the type of cells used (primary hepatocytes or transfected HEK293), MC-RR was always 1–2 orders of magnitude less cytotoxic than MC-LR.

The different toxicities observed among MC variants could be attributed to their minor structural changes, as pointed out earlier. Thus, MC-RR is more polar than MC-LR and therefore is not as easily transported via the bile acid transport system as MC-LR (see Section 3). Moreover, the dissimilar possibility of entering a cell would explain the different levels and toxic effects observed in tissues for MC congeners (Amé et al., 2010). Thus, Atencio et al. (2008) found that, although MC-RR was hepatotoxic in i.p. exposed fish, the organ most affected was the kidney.

A review of the main toxicological studies focused on MC-RR or extracts, MC-RR being the most abundant congener, is provided below.

4.2. In vitro toxicity studies

4.2.1. Cytotoxicity studies

Table 3 shows the various in vitro assays that have been performed with MC-RR between the years 2000 and 2017. Due to MC-RR hepatotoxicity different studies have been carried out on hepatic cell lines (PLHC-1, H4IIE.luc, HepG2, primary hepatocytes, etc.). Moreover, as

MCs can enter organisms through consumption of contaminated food and water, other studies have been performed on intestinal cell lines. However, it has been also demonstrated that MC-RR affects several organs and tissues. Thus, there are also studies on other cell lines, such as RTG-2 from gonads, P19 from embryo, HaCat from keratinocytes, etc. All these studies have been performed on cell lines from animals (including fish), mammals, and humans. However, it is also noteworthy that a number of studies have been carried out on the tobacco BY-2 cell line, which demonstrated the negative effects that MC-RR could also induce in plants.

With regard to animal cells, Puerto et al. (2009) showed that undifferentiated and differentiated Caco-2 cells exposed to 50–200 μM MC-RR experienced a time- and concentration-dependent reduction of cell viability, which never reached a mean effective concentration (EC_{50}) value, with no clear differences between the two cell types. Also, Zeller et al. (2011) showed no cytotoxic effects in undifferentiated Caco-2 cells after 24 h of exposure to MC-RR. In agreement with these authors, Huguet et al. (2013) showed a reduction of 25% of cell viability in differentiated Caco-2 cells exposed to 0–100 μM MC-RR after an exposure of 24 h. These results indicate that the sensitivity of the Caco-2 cell line to MC-RR was low. However, this was not the case in hepatic cells, where cytotoxic effects were observed at low concentrations of MC-RR. In this regard, the most sensitive experimental models were human primary hepatocytes, with EC_{50} values between 0.056 and 900.2 nM MC-RR (Ikehara et al., 2009; Fischer et al., 2010). However, Ufelmann et al. (2012) reported contradictory results, as concentrations up to 100 nM did not show toxicologically relevant effects in these human cells, which could be due to the exposure time used (24 h, instead of the 48 h and 72 h used by Fischer et al. and Ikehara et al., respectively). These results agreed with those obtained in rat hepatocytes by Shimizu et al. (2013), who demonstrated an absence of cytotoxic effects when cells were exposed to 0–10 μM MC-RR. Finally, Pichardo et al. (2005) evaluated the cytotoxicity induced by this cyanotoxin in the fish hepatic cell line PLHC-1, reporting an EC_{50} value of 79 μM MC-RR, the highest EC_{50} value shown in Table 3, which could be due to the adaptive response presented by fish to aquatic contaminants, as Gutiérrez-Praena et al. (2011) stated for the same cell line exposed to another cyanotoxin.

With respect to other cell lines, it should be noted that studies carried out in reproductive and embryo cell lines did not show cytotoxic effects at low MC-RR concentrations in HeLa and P19/A15 cells (Chen et al., 2014; Javůrek et al., 2015). However, at higher MC-RR concentrations Chen et al. (2014) found cell apoptosis, and Pichardo et al. (2005) observed cytotoxic effects for the RTG-2 cell line. On the other hand, Monks et al. (2007) showed that HeLa cells transfected with the organic anion transporting polypeptides OATP1B1 and 1B3 increased the sensitivity of this cell line, reporting EC_{50} values of 3.8 and 0.58 μM MC-RR, respectively. This highlights the importance of the presence of OATPs in the cell line, because they contribute to uptake of the toxin. It also agrees with the results presented by Fischer et al. (2010), who found that the renal HEK293 cell line transfected with the same OATPs presented an EC_{50} value of 1.27 μM for cells transfected with OATP1B3. Cells transfected with OATP1B1 appeared to be more sensitive, which is also in agreement with the results presented by Monks et al. (2007). However, this cell line seemed to be more resistant to the effects of MC-RR compared to the HeLa cell line. This type of study brings to light the importance of identifying and knowing the various polypeptides involved in the transport of MCs to cells. It is also interesting to note the resistance shown by the keratinocyte cell line HaCat, which presented an EC_{50} very high (57 mM MC-RR) (Štěpánková et al., 2011), possibly because keratinocytes could constitute one of the first barriers against the action of toxicants.

In plants, cytotoxic effects of MC-RR have been evaluated in the tobacco BY-2 cell line (0 and 120 μg/mL). The lowest concentrations that induced cytotoxic effects were assayed by Huang et al. (2008a), and they presented a time- and concentration-dependent reduction of cell viability. However, Yin et al. (2005b) found a slight reduction of cell viability in cells exposed to 50 μg/mL for up to 144 h, after which a recovery of viability was observed. The same authors found that MC-RR induced cell apoptosis from 1 μg/mL to 50 μg/mL (Yin et al., 2006), which is in agreement with their previous viability studies. In addition, various studies carried out by Huang et al., (2008b, 2009, 2014) found that 60 μg/mL MC-RR induced cell apoptosis and 120 μg/mL MC-RR induced cell necrosis.

In general, animal cell lines appear to be more sensitive to MC-RR than cells from plants, with the exception of the RTG-2 and PLHC-1 fish cell lines and the HaCat keratinocyte cell line, possibly for the same reasons as those given earlier. Plants may present a defensive mechanism against aquatic toxins because they are continuously exposed to them from water reservoirs and irrigation water.

With regard to the biomarkers considered, most of the reports used basal cytotoxicity/cell viability tests (such as Neutral Red assay, MTS reduction, etc.), although some of them also studied cell death mechanisms, particularly apoptosis (i.e. Yin et al., 2006; Zhang et al., 2006; Chen et al., 2014, etc.). The apoptotic damage has also been confirmed by morphological changes identified by microscopy. Additionally, a general reduction in cell numbers, steatosis, and hydropic degeneration were observed (Pichardo et al., 2005; Puerto et al., 2009). Thus it is well established that MC-RR has a great potential to induce cytotoxic effects.

Compared with other MCs congeners, MC-RR and its variants appeared to be the less cytotoxic. Thus, the cytotoxicity rank observed by Ikehara et al. (2009) in human normal hepatocytes was MC-RR < MC-YR < MC-WR < MC-LR < MC-LA. Differences of cytotoxicity between MC-LR and MC-RR was also observed by Huguet et al. (2013) in differentiated Caco-2 cells. Puerto et al. (2009) also found in the same cell line that MC-RR was less toxic than the congener MC-YR.

4.2.2. Oxidative stress studies

Oxidative stress has been described as a toxic mechanism of MCs (Campos and Vasconcelos, 2010). As can be seen in Table 4, this mechanism has scarcely been studied in vitro, and some authors have also pointed out that studies of this kind are scarce in animal cells (Lundqvist et al., 2017). Actually, there are only 2 studies carried out in animal cell lines, one on fish lymphocytes and another one in human Caco-2 cells. However, some studies using these cells have focused on proteomic and genomic analysis of them in order to study the proteins and genes affected by MC-RR exposure. Thus, Fu et al. (2009) carried out a proteomic analysis of human amniotic epithelial cells (FL cells) exposed to MC-RR, reporting that some antioxidant enzymes, such as superoxide dismutase (SOD), presented a different expression compared to the control group. Also, among all the genes included in a transcriptomic study of Caco-2 cells exposed to different concentrations of MC-RR, Zeller et al. (2012) found some alterations in oxidative stress genes. In addition, Lundqvist et al. (2017) showed that MC-RR affected expression of erythroid 2-related nuclear factor (Nrf2), which is a key regulator of oxidative stress response, in human liver cells (HepG2 cells).

With regard to plants, the tobacco BY-2 cell line was the experimental model most frequently used. MC-RR has been shown to induce oxidative stress in plants through a time- and concentration-dependent increase in reactive oxygen species (ROS) levels in most of the studies presented in the table. Some authors point out that ROS and mitochondria are involved in cell apoptosis (Huang et al., 2008b, 2014). More specifically, Huang et al. (2014) linked MC-RR-induced ROS generation with the mitochondrial electron transport chain (ETC), because when the ETC was inhibited the ROS levels did not differ from those of the control group. Moreover, these authors demonstrated that ROS derived from ETC-induced apoptosis acted as a signaling molecule in plants. In addition to ROS generation, Yin et al. (2005b) described a time- and concentration-dependent increase in lipid peroxidation (LPO) when cells were exposed to MC-RR, showing the role of this cyanotoxin

Table 4
In vitro oxidative stress studies performed with MC-RR.

Toxin	Experimental model	Assays Performed	Concentration range and Exposure Conditions	Main results	Reference
Purified MC-RR extract from *Microcystis aeruginosa*	BY-2 cells (tobacco)	ROS SOD POD CAT GPx	50 mg/L for 6 days	A time-dependent increase of ROS levels was observed, being significant respect to the control from day 2. Basal levels where reached after a recovery time (1 week). A time-dependent increase of the activity of antioxidant enzymes was observed, except for SOD, which experienced a decreased from day 2.	Yin et al. (2005b)
Purified MC-RR extract from *M. aeruginosa*	*Arabidopsis thaliana* cells	LPO SOD CAT GSH GST	0–5 mg/L for 24 and 48 h	No parameter was affected after 24 h of exposure. After 48 h, LPO significantly increased respect to control from 1 mg/L. GSH levels significantly decreased from 0.1 mg/L. CAT and SOD activities significantly increased from 1 mg/L. GST activity did not suffer any change at any concentration assayed.	Yin et al. (2005c)
Purified MC-RR extract	*Synechococcus elongatus*	ROS LPO GPx GSH GST	100 μg/L for 6 days	Both ROS and LPO levels increased exponentially from day 1 to day 6. GSH content significantly increased from day 3. Both antioxidant enzymes presented a decrease of their activity at the beginning of the experiment, although from day 3 a significant increase of the activity of both enzymes was observed.	Hu et al. (2005)
Purified MC-RR extract from *M. aeruginosa*	BY−2 cells (tobacco)	ROS GPx GR GSH/GSSG GST	0–10 μg/mL for 6 days	A time- and concentration-dependent increase of ROS levels was observed, being significant respect to the control from day 3. GSH levels increased with time, although at day 6, its levels decreased, coinciding with an increase of GSSG. The activities of GPx, GST and GR increased with time and concentration till day 4. At day 6, GST and GR activities suffered a decrease respect to the control, while GPx activity increased markedly.	Huang et al. (2008a)
Purified MC-RR extract from *M. aeruginosa*	BY−2 cells (tobacco)	ROS	60 μg/mL for 5 days	A time-dependent increase of ROS levels was observed, being significant respect to the control from day 2, reaching the highest levels on day 4.	Huang et al. (2008b)
Pure MC-RR commercial standard	Lymphocytes from *Carassius auratus*	ROS	10 nM for 6 h	ROS levels increased gradually with time till 3 h. From this time, ROS decayed with time.	Zhang et al. (2008)
Purified MC-RR extract from *M. aeruginosa*	*Synechocystis* sp. PCC6803	*sodB*, *gpx1*, *γ-TMT*, *16sRNA*, *katG*, *acnB*, *dnaK2* by Q-rtPCR	0–5 mg/L for 24 h	Different genes related directly or indirectly with oxidative stress were evaluated. An up-regulation from day 2–6 of all genes implicated was observed when cells were exposed to the highest concentration of MC-RR, indicating the activation of the defense mechanisms against the oxidative damage induced by the cyanotoxin.	Li et al. (2009)
Pure MC-RR commercial standard (and MC-LR)	Differentiated Caco−2 cells	ROS	0–100 μM for 4 and 24 h	MC-RR induced a time- and concentration-dependent increment of ROS levels, being significant respect to the control group from the lowest concentration assayed (1 μM).	Huguet et al. (2013)
Purified MC-RR extract from *M. aeruginosa*	BY−2 cells (tobacco)	ROS	60 mg/L for 6 days	A significant ROS increase was observed after 3 and 6 days of exposure to the toxin. The presence of the mitochondrial electron chain inhibitors reduced the presence of ROS.	Huang et al. (2014)

Abbreviations: BY-2 cell line: *Nicotiana tabacum* L. cv. Bright Yellow 2; Caco-2: Human colorectal adenocarcinoma cell line; CAT: catalase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione; GST: glutathione S-transferase; LPO: lipid peroxidation; POD: peroxide dismutase; Q-rtPCR: quantitative real-time PCR; ROS: reactive oxygen species; SOD: superoxide dismutase.

Table 5
In vivo toxicological studies dealing with MC-RR exposure.

Toxin/ Cyanobacteria	Experimental model	Organs Studied	Assays performed	Exposure conditions Concentration ranges	Main results	Reference
Commercial MC-RR pure standard (and MC-LR).	Frog embryo-larvae *(Xenopus laevis)*	Larva homogenates	Mortality Malformation Growth inhibition Protein and PP-1 and PP2A activity	1, 10, 100, 500 and 2000 μg/L for 24, 48, 72, 96 and 120 h.	No mortality, malformation or growth inhibition were observed. PP1 and PP-2 are present in early life-stages *of X. laevis*. However, only after 96 h MC-RR induced a significant inhibition of PP activity at 2000 μg/L. (MC-LR at 120 h).	Fischer and Dietrich, (2000).
Commercial MC -RR pure standard (and MC-LR and MC-YR).	Swiss albino female mice	Liver Lung	LBI Serum activity of: ALT, AST, g-GT, TP, ALB, Globulin A/G, LDH, hepatic GSH. DNA fragmentation. Respiratory variables. Liver and lung histology	Single LD_{50} dose of MC-RR: 235.4 μg/kg bw Variables determined at 30 min post-treatment and mean time to death (MTD)	The toxin showed significant increase in LBI at MTD. Increases in AST, ALT, g-GT, LDH and DNA fragmentation at 30 min post-treatment were observed and were further enhanced at MTD. MC-RR caused depletion of hepatic GSH levels, although lower in comparison to MC-LR. No significant changes in total protein, ALB and A/G ratio were observed. Histological changes in the liver (congestion and hemorrhage, predominantly) and in the lung (bronchial epithelial necrosis) were observed. The breathing pattern and respiratory variables showed uniform pattern for 90 min followed by abrupt change in the respiratory pattern and instantaneous death.	Gupta et al. (2003).
Purified extract containing mainly MC-RR (and MC–LR and MC-YR)	Bighead carp (*Aristichthys nobilis*)	Liver	Determination of MC concentration in liver by LC-MS Ultrastructural changes	Extract containing MC-RR 0.84 mg/g DW (dry weight), MC-LR 0.50 mg/g DW and MC-YR 0.07 mg/g DW Two doses equivalent to 200 and 500 MC-LR μg/Kg bw for 1, 3, 12, 24 and 48 h were ip-injected.	MC concentration in the liver reached the maxima at 3 h (at the higher dose) or at 12 h (at the lower dose) post-injection. The ultrastructural changes observed were similar with characters of cell apoptosis (200 μg MC-LR/kg) and necrosis (500 μg MC-LR/kg). However, remarkable decrease or even disappearance was evident in both dose groups in the liver at 48 h. MC act firstly on the intercellular junctions of hepatocytes in fishes.	Li et al. (2005a).
Purified extract containing mainly MC-RR	*Misgurnus mizolepis*	Liver	LPO, SOD, CAT, GPx,	10 μg MC-RR/kg for 28 days orally exposed	SOD, CAT and GPx activity were significantly increased. No significant changes in LPO were observed.	Li et al. (2005b).
Commercial MC-RR pure standard	*Corydoras paleatus*	Liver Gill Intestine Brain	LPO, CAT, GR, POD, GPx, GST,	0.5, 2, 5 and 10 μg/L for 24 h (dissolved in aquarium water)	Exposure to MC-RR caused a reduction of GST in all studied organs. GR, POD, GPx, CAT activities were significantly increased in liver at 2 μg/L, but in gill, GR, POD and GPx were inhibited at all tested concentrations. LPO was enhanced in brain (≥ 2 μg/L), but significantly reduced in liver and gills. No significant changes in GR and LPO in intestine were detected	Cazenave et al. (2006a).
Commercial MC-RR pure standard (and MC-LF)	Embryos Zebrafish *(Danio rerio)*	Fish embryo	Embryonic development (retardation in development and deformations, mortalities).	MC-RR: 25 μg/L or MC-LF: 25 μg/L + Natural organic matter (NOM) incubated in Petri dishes for:	MC-RR caused lower teratogenic effects than MC-LF, despite the higher mortalities caused by MC-RR.	Cazenave et al. (2006b).

(*continued on next page*)

Table 5 (*continued*)

Toxin/ Cyanobacteria	Experimental model	Organs Studied	Assays performed	Exposure conditions Concentration ranges	Main results	Reference
					GSTs and CAT activities were significantly increased in embryos exposed to MC-RR. Pure MC-RR caused lower H_2O_2 formation than pure MC-LF. Carbohydrates and proteins showed no significant changes, and a reduction in lipid content was observed.	
				12, 14, 36 and 48 h →embryotoxicity24 h →enzymatic studies, H_20_2 content and evaluation of the energetic costs		
			GSTs, CAT, POD, GPx, GR, H_20_2 content. Carbohydrates, proteins and total lipids.			
MC-RR isolated from freeze-dried crude algae (and MC-LR, MC-FR, MC-WR, [D-Asp^3]MC-FR, -WR, -RR and [Dha^7]MC-RR)	Mouse	-	Acute toxicity test (LD_{50})	Pure MC (1.5–36 mg) was dissolved in saline solution at different concentrations and 1 ml toxin solution was ip-injected for 4 h.	The least toxic MC was MC-RR and its derivatives, with LD_{50} values ranging from 320 to 420 μg/kg. MC-LR has the lowest LD_{50} value (50 μg/kg).	Chen et al., (2006)
Commercial MC-RR pure standard (and MC-LR)	Tilapia fish (*Oreochromis* sp.)	Liver Kidney Gill	LPO, SOD, CAT, GPx, GR	Single dose of 500 μg MC-RR/kg ip-injected and after 7 days of exposure the fish were sacrificed.	Mainly, SOD, CAT activity and LPO increased significantly after acute exposure to MC-RR. GPx activity was only increased in the kidney by MC-RR, and no significant changes of the basal levels of GR activity were detected in any of the organs studied.	Prieto et al. (2006).
Purified extract containing mainly MC-RR (and –LR)	Silver carp (*Hypophthalmichthys molitrix*)	Liver	Determination of MC concentration in liver by LC-MS. ALT, AST, LDH, GST, GR, GSH.	Extract containing MC-RR: 0.84 mg/g DW, MC-LR: 0.50 mg/g DW and MC-YR: 0.07 mg/g DW	MC concentration in the liver reached the maxima at 3 h and rapidly dropped at 48 h. A significant increased in ALT, AST and LDH were observed.	Li et al. (2007b).
			Ultrastructural changes	A dose equivalent to 1000 MC-LR μg/kg bw for 1, 3, 12, 24 and 48 h were ip-injected.	GST activity increased significantly at 24 and 48 h post-injection. There were no significant changes in GR activity and GSH level. Lysosomes proliferation, vacuolized mitochondria and ER were the mainly histopathology changes observed.	
Commercial MC-RR pure standard (and MC-LR)	Tilapia fish (*Oreochromis* sp.)	Liver Kidney Gill Heart	ACP, ALP. Histopathological study	Single dose of 500 μg MC-RR/kg ip-injected and after 7 days of exposure the fish were sacrificed.	ACP and ALP activity increased significantly in the kidney and liver of fish injected with MC-RR, respectively. The pathological changes in the kidney of the group treated with MC-RR were more severe than those in the MC-LR group. The main histological findings in the kidney were glomerulopathy with alteration of the basal membrane.	Atencio et al. (2008).
Purified extract containing mainly MC-RR (and –LR)	Rabbit (*Oryctolagus cuniculus*)	Liver Heart	Mitochondrial morphology LPO, SOD, NADH dehydrogenase, SDH, ATPase activity	Two doses of 12.5 and 50 MC-LReq.μg/kg bw and sacrifice at 1, 3, 12, 24 and 48 h after ip-injection.	MC from the extract induced ultrastructural changes in mitochondria, LPO and significant increase of SDH activity in liver and heart. At 3 h post-MC exposure, liver mitochondrial NADH dehydrogenase activity was decreased. SOD activity increased significantly in hepatocytes and cardiocytes. However, in the high dose group, reduction in SOD activity in cardiocytes was observed at 3 h before their death. MC altered Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities of mitochondria in hepatocytes and cardiocytes.	Zhao et al. (2008).
Commercial MC-RR pure standard	Embryos zebrafish (*Danio rerio*)	Zebrafish embryos	Survival and malformation rates	Different doses of MC-RR by microinjection at a range of 0.2 LD_{50} (7.2 μM), 0.4 LD_{50} (14.4 μM) and 0.8 LD_{50} (28.8 μM). Observations at 24, 48	MC-RR decreased dose-dependently the survival rates and increased malformation rates.	Zhao et al. (2011).

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Table 5 (*continued*)

Toxin/ Cyanobacteria	Experimental model	Organs Studied	Assays performed	Exposure conditions Concentration ranges	Main results	Reference
			2-DE analysis MS analysis of proteinsmiRNA microarray	and 72 hours post fertilization. The LD_{50} = 36 μM.	MC-RR changed expressions of 31 miRNAs at 72 h, such as miR-430 and miR-125 families. The expressions of a number of proteins involved in energy metabolism, cell division, protein synthesis, cytoskeleton maintenance, response to stress and DNA replication were significantly reduced.	
Commercial MC-RR pure standard (and MC-LR)	Adult Zebrafish (*Danio rerio*)	Liver Gill Intestine Brain	SOD, GPx, GR, GST	0.1, 0.5, 1, 5 and 10 μg/L for 4, 7 and 15 days (dissolved in aquarium)	Exposure to MC -RR induced perturbations in the antioxidant activities of different tissues studied. Enzyme activities in general increased at lower concentrations (≤ 5 μg/L) and decreased at higher concentrations (≥ 5 μg/L). There was a differential response in the case of fish exposed to MC-RR and -LR for GPx and GR activity.	Pavagadhi et al. (2012).
MC-RR isolated from freeze-dried crude algae	Adult Zebrafish (*Danio rerio*)	Testes	Acute toxicity (LD_{50}) LPO, SOD,CAT, GST, GPx, GSH content, H_20_2 content. Ultrastructural changes Proteomic and western blot analysis	A dose of 0.5 LD_{50} (2000 μg/kg) ip-injected. Three sampling points were set during a period of 24 h (2, 6 and 24 h).	24 h LD_{50} of MC-RR (i.p.) was calculated to be about 4000 mg MC-RR/kg BW. LPO, H_20_2 and GSH content, and the activities of antioxidant enzymes significantly increased after injection of MC-RR. MC-RR was able to damage the testes. 24 proteins were identified as implied in toxic mechanisms of MC-RR and were related with: 1) cytoskeleton assembly (tubulin and keratin); 2) oxidative damage (as heat shock protein and aldehyde dehydrogenase); 3) glycolysis metabolism (aldolse B 23 kDa protein, phosphoglycerate mutase, enolase, citrate synthase and nucleoside diphosphate kinase-Z2); 4) calcium ion binding (as calmegin); and 5) other biological functions.	Zhao et al. (2012).
MC-RR isolated from freeze-dried crude algae	Goldfish (*Carassius auratus*)	Liver Kidney Heart	TAOC, Glucose, SOD, CAT, GPx. mRNA expression of myoglobin 1 (Myb1), myoglobin 2 (Mb2), cytoglobin (CYb) and neuroglobin (Ngb) Ultrastructural changes	50 and 200 μg/kg bw ip-injected under normoxia, hypoxia and reoxygenation.	SOD activity increased in liver and kidney at both doses, but TAOC (liver, heart and brain) and CAT in liver were low during hypoxia and reoxygenation compared to normoxia and control. Myb1, Mb2 and Ngb were significantly induced in the brain only. Extensive damage as vacuolization mitochondria and cytoplasm was observed in the liver cells.	Okogwu et al. (2014).
Commercial MC-RR pure standard	Zebrafish larvae	Zebrafish larvae	Survival rate, growth and heart rate. T4 and T3 content. Gene expression of CRF, transcription of the TSHβ, TG, the NIS, thyroid receptors (TRα and TRβ), and iodothyronine deiodinases (Dio1 and Dio2). Protein expression of TG and NIS.	0, 0.3, 1 and 3 mg/L for 96 h	MC-RR induced growth delay, depressed heart rates and decreased levels of THs. The gene expression of CRF, TSHβ, NIS, TRα and TRβ, Dio1 and Dio2 were greatly changed by MC-RR exposure, except TG gene. In this sense, expression of TG was not affected, while that of NIS was significantly up-regulated in 1 and 3 mg/l MC-RR.	Xie et al., (2015).
Purified extract from *Microcystis aeruginosa* containing MC-RR	Adult KM mice	Liver Kidney	Histological studies. Water intake and urine output.	1) 0, 35, 70 and 140 μg MC-RR /kg administered daily for a month: biochemical and toxicological analyses.	140 μg/kg of MC-RR induced weight loss and liver damage, although it had no adverse impacts on the kidneys.	Zhong et al. (2017).

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Table 5 (*continued*)

Toxin/ Cyanobacteria	Experimental model	Organs Studied	Assays performed	Exposure conditions Concentration ranges	Main results	Reference
			USG, ALT, AST, ALP,TP, ALB, UA, Glu, TC, BUN, CREA, Levels of serum AGT, Ang II, AVP. Gene expression of AGT and ACE in liver.	2) 0, 35, 70 and 140 μg MC-RR /kg administered daily for a month and the recovery time was extended to 12days (the water metabolism recovery process) and to 42 days. Blood and urine samples were collected on days 7, 14, 21, 28, 35 and 42. 3) three groups of animals were treated daily for 30 days with dosages of MC-RR 140 μg/kg BW + aliskiren 100 mg/kg BW, MC-RR 140 μg/kg BW + aliskiren 50 mg/kg BW, and MC-RR 140 μg/kg BW + physiological saline.	The highest dose caused an increase in water intake and urine output. However, MC-RR + 100 mg/kg of aliskiren inhibited these effects. The urine specific gravity decreased and ALT, TC, ALP, AST increased in the high-dose group compared with the control. The mRNA level of AGT and ACE in hepatocytes was upregulated to approximately 150% of the control. The RAS was activated in the high-dose group.	

Abbreviations: ALB: Albumin; A/G: Albumin/globulin ratio; ACE: Angiotensin-converting enzyme; ACP: Acid phosphatases; AGT: Angiotensinogen; ALP: Alkaline phosphatase; ALT: Alanine amino transferase; Ang II: angiotensin II; AST: Aspartate aminotransferase; AVP: arginine vasopressin; BUN: Urea nitrogen; CAT: Catalase; CREA: Creatinine; CRF: Corticotropin-releasing factor; CYb: Cytoglobin; Dio1 and Dio2: Iodothyronine deiodinases; DW: dry weight; ER: endoplasmic reticulum; Glu: Glucose; g-GT: g-glutamyl transpeptidase; GPx: glutathione peroxidase; GR: Glutathione Reductase; GSH: Glutathione; GST: Glutathione-S-transferase; LBI: Liver body weight index; LD_{50}: Lethal dose 50; LDH: Lactate dehydrogenase; LPO: Lipid peroxidation; MTD: Mean Time to Death; MS: Mass spectrometer; Myb1: Myoglobin 1; Mb2: Myoglobin 2; Ngb: Neuroglobin; NIS: Sodium/iodide symporter; POD: guaiacol peroxidase; PP-1: Protein phosphatases-1; PP-2A: Protein phosphatases-2A; POD: Guaiacol peroxidase; RAS: Serum renin-angiotensin system; SDH: Succinate dehydrogenase; SOD: Superoxide dismutase; TAOC: Total antioxidant capacity; TC: Total cholesterol; TG: thyroglobulin; TP: Total protein; TSHβ: thyroid-stimulating hormone; TRα -β: Thyroid receptors; 2-DE: Two-dimensional gel electrophoresis; UA: Uric acid; USG: Urine specific gravity.

in the oxidative status of plants. This LPO increase could be derived from a failure of the antioxidant system to eliminate the excess of ROS (Yin et al., 2005b). With regard to glutathione (GSH) levels, there were contradictory results when cells were exposed to similar MC-RR concentrations, as some authors found that GSH levels decreased with time and concentration (Yin et al., 2005b), while other authors found an increase in GSH levels when cells were exposed to MC-RR (Huang et al., 2008a). This could be explained by direct scavenging of ROS, enhancement of the activity of the enzyme glutathione reductase (GR), or an increase in GSH synthesis. In relation to antioxidant enzymes, all the studies report changes in the antioxidant system when plant cells were exposed to MC-RR. Some enzymes increased their activity when cells were exposed to MC-RR, and some enzymes suffered a decrease. This could be explained by taking into account the previous redox status of the cells, as has been pointed out (Yin et al., 2005a). Moreover, it is also noteworthy that some of the parameters studied recovered with time, which implies an adaptive response of plant cells to oxidative damage (Huang et al., 2008b).

With regard to cyanobacterial cells, Hu et al. (2005) showed that ROS and LPO levels increased with time after exposure to 100 μg/L MC-RR in the cyanobacterium species *Synechococcus elongatus*, which is in agreement with the results in BY-2 cells. These authors also found that GSH levels decreased and increased, and the changes were not time-dependent. The authors explained the GSH level depletion as being due to the involvement of this molecule in other processes apart from ROS detoxification. The increase in GSH level was explained by assuming that the algae presented a sensitive response to the toxin. This is in agreement with the findings of Li et al. (2009), who reported that, by performing a Q-rtPCR in *Synechocystis* sp. cells, expression of the *dnaK2* gene (encoded for Hsp70), involved with GSH levels, was upregulated. With respect to antioxidant enzymes, Hu et al. (2005) reported enhancement of activity with time, so it seems clear that cyanobacteria possess an adaptive response to MC-RR. This was corroborated by Li et al. (2009), who found that expression of the *gpx1* gene (encoded for GPx) was upregulated at the beginning of the exposure time. However, this gene was downregulated at the midpoint of the exposure time, although the authors related this to the regulation of other antioxidant genes, such as *sodB* (encoded for FeSOD) and *katG* (encoded for CAT peroxidase), suggesting that there was a coordinated expression of enzymes for defense against ROS induced by MC-RR.

With regard to the oxidative stress biomarkers assayed, the study of ROS levels is the common point in almost all the cases presented in Table 4, which highlights the importance of this endpoint no matter which experimental model is employed. This also agrees with the alterations of the activity of the various antioxidant enzymes, although they do not always show the same response. This could be due, as Hu et al. (2005) pointed out, to different sensitization mechanisms in the different cell lines. Nevertheless, the almost complete lack of studies on oxidative stress in human cell lines is noteworthy.

In general, as has been shown above, most of the in vitro studies performed with MC-RR in cells deal with cytotoxicity (the starting point of any toxicological evaluation) and oxidative stress (as this is a well-known mechanism of toxicity). However there are other toxicological aspects that can be investigated using in vitro cellular experimental models (mutagenicity, genotoxicity, immunotoxicity, etc.) and that have not yet been sufficiently addressed with pure or purified MC-RR. Given that pure MC-LR or cyanobacterial extracts are able to induce these kinds of effects (i.e. Lone et al., 2016, for immunotoxic effects, Sieroslawska (2013), for mutagenic potential), it cannot be ruled out that MC-RR may also be able to do so. Therefore, further research is needed to fill these data gaps.

The lack of literature concerning the oxidative stress induction of MCs in vitro does not allow to perform a comparative analysis of the effects induced by MC-RR and other MCs variants. Only Huguet et al. (2013) compared the effects induced by MC-LR and MC-RR, concluding that MC-LR presented a higher ROS generation capacity than MC-RR,

although these differences were not significant.

4.3. In vivo toxicity studies

With regard to in vivo studies, various reports have been published from 2000 up to the present time (Table 5). Most of them focus on aquatic organisms (Atencio et al., 2008; Cazenave et al., 2006a, 2006b; Li et al., 2005a, 2005b, 2007b; Prieto et al., 2006; Pavagadhi et al., 2012; Okogwu et al., 2014; Xie et al., 2015; Fischer and Dietrich, 2000; Zhao et al., 2011, 2012), as MCs are produced mainly in aquatic environments. By contrast, very little research has been carried out in mammals, such as mouse (Gupta et al., 2003; Zhong et al., 2017) and rabbit (Zhao et al., 2008). In general, the data indicate that the biomarkers most assayed were antioxidant enzymes, with histopathological changes also being reported in most of the studies (Cazenave et al., 2006a, 2006b; Gupta et al., 2003; Li et al., 2005a, 2005b, 2007b; Prieto et al., 2006; Zhao et al., 2008, 2012; Atencio et al., 2008; Pavagadhi et al., 2012; Okogwu et al., 2014; Zhong et al., 2017). To our knowledge, there are two studies that examines the potential embryotoxic effects of MC-RR (Fischer and Dietrich, 2000; Zhao et al., 2011), only one dealing with its activity as an endocrine disrupter (Xie et al., 2015), and one that investigated its effects on water metabolism dysfunction via the rennin–angiotensin system (Zhong et al., 2017). Regarding embryotoxic effects, microRNA expression has been analyzed in zebrafish embryos exposed to MC-RR (Zhao et al., 2011), and proteomic analyses have been performed in testes of zebrafish (Zhao et al., 2012). Although several studies focus on the toxic effects induced by pure MC-RR commercial standard, there are in vivo toxicological studies that have been performed with purified extracts of cyanobacteria containing mainly MC-RR but also MC-LR, and consequently the effects observed were not exclusively due to MC-RR (Li et al., 2005a; b; Li et al., 2007b; Zhao et al., 2008; Zhong et al., 2017). A summary of the most important findings of the in vivo studies performed with MC-RR follows.

In aquatic models, several works demonstrated the role of oxidative stress as a toxic mechanism of MC-RR. In this regard, MC-RR exposure induced adaptive responses, such as an increase in antioxidant enzymatic activities in liver of fish, mainly SOD (Okogwu et al., 2014) and catalase (CAT) (Cazenave et al., 2006a, 2006b; Prieto et al., 2006). Cazenave et al. (2006a) found a dual response of CAT activity in the liver of fish after MC-RR exposure, with an increase at low concentrations (≤ 2 μg/L) and a depletion at higher levels (≥ 5 μg/L). Similarly, in adult *Danio rerio* (zebrafish) exposed to sublethal concentrations of MC-RR, the enzyme activities generally increased at ≤ 5 μg/L and decreased at ≥ 5 μg/L (Pavagadhi et al., 2012). The reduced activity could be attributed to secondary effects due to substrate inhibition by covalent modification of the enzyme. On the other hand, a significant inverse relationship was observed between SOD and CAT activities in the liver and kidney of goldfish exposed to MC-RR and reoxygenation treatments, which could indicate that SOD activities may directly or indirectly inhibit CAT activity (Okogwu et al., 2014). Cazenave et al. (2006b) also reported higher glutathione-S-transferase (GST) activities with 25 μg/L of MC-RR in zebrafish embryos, suggesting that the biochemical response of adult fish is quite similar to that of the embryos with respect to the GST profile. Only a few studies reported oxidative stress responses in kidney of fish exposed to MC-RR (Prieto et al., 2006; Okogwu et al., 2014). Prieto et al. (2006) observed more significant increases in SOD, CAT, and glutathione peroxidase (GPx) activities in kidney of tilapias after acute exposure to pure MC-RR, in comparison to the liver or gills. The higher susceptibility of the kidney to MC-RR exposure could be related to its higher hydrophilicity in comparison to MC-LR, which facilitates its distribution to this organ. For GPx and GR, wide variations in their activities after various toxin exposure times have been reported in several studies, and a differential response in zebrafish exposed to MC-RR and MC-LR has been demonstrated (Pavagadhi et al., 2012). According to these authors, the differences between the two toxins at the same point in the same tissue could be explained by their toxicity potentials. Thus, although MC-RR is less toxic than MC-LR, it produces a more rapid adaptive response than MC-LR. GST enzymes, involved in the detoxification of MCs, could suffer variations after MC-RR exposure. Thus, an increase in GST activity was observed in liver, intestine, gill, and brain of adult zebrafish after 7 and 15 days of exposure to MC-RR, suggesting an adaptive response by the tissues to continued exposure to the toxin (Pavagadhi et al., 2012). By contrast, a dose-dependent reduction of GST activity was observed in liver and brain of fish exposed to high concentrations of MC-RR.

The increased enzymatic activities observed could contribute to reducing LPO levels, as was observed in liver and gills of fish (Cazenave et al., 2006a). In contrast, this parameter was not affected in the intestine of *Corydoras paleatus* exposed to MC-RR dissolved in aquarium water at concentrations of 0.5–10 μg/L for 24 h (Cazenave et al., 2006a), or in the liver of loach orally exposed to *Microcystis* (10 μg MC-RR/kg) for 28 days (Li et al., 2005b). Globally, however, MC-RR induced an increase of LPO in liver, kidney, and gills of tilapia injected i.p. with a single dose of 500 μg/kg and sacrificed 7 days later (Prieto et al., 2006), in brain of fish exposed to 2–10 μg/L MC-RR (Cazenave et al., 2006a), and in testes of zebrafish after an i.p. injection with 2000 μg/kg MC-RR (Zhao et al., 2012). On the other hand, the combined effects of MC-RR (i.p.) and hypoxia-reoxygenation transitions have been evaluated in goldfish, and neuroglobin (Ngb) and myoglobin (Myb1 and Mb2) mRNAs were significantly induced in the brain only, suggesting that these globins may protect this organ against oxidative stress (Okogwu et al., 2014).

In relation to histopathological changes, evidence of liver damage induced in fish by MC-RR includes time- and dose-dependent ultrastructural effects (Li et al., 2005a, 2007b). Among the early ultrastructural changes induced, Li et al. (2005a) found widening of intercellular spaces. Moreover, swollen and vacuolized mitochondria and endoplasmic reticulum, necrosis of hepatocytes with lysosome and ribosome proliferation, cytoplasmic vacuolization, and lipid droplets were observed in the liver of tilapias acutely exposed (i.p.) to pure MC-RR (Atencio et al., 2008), and in goldfish exposed simultaneously to MC-RR and hypoxia-reoxygenation transitions (Okogwu et al., 2014). In tilapia, MC-RR also caused severe pathological lesions in kidney (degenerative renal changes and glomerulopathy) and heart (fibrolysis and edema) (Atencio et al., 2008). The greater affectation of kidney could be related to the higher hydrophilicity of MC-RR, as stated by Prieto et al. (2006). Moreover, in comparison to MC-LR, although the lesions caused by both congeners were qualitatively identical, there were quantitative differences: MC-LR caused more hepatotoxic damage, whereas MC-RR toxicity was more severe in the kidney (Atencio et al., 2008). The ultrastructural changes may indicate the presence of an adaptive mechanism to eliminate or ameliorate cell damage caused by MC-RR (Li et al., 2007b). Li et al. (2005a) demonstrated that hepatocyte damage in fish tends to proceed toward the direction of apoptosis (at lower toxin concentrations) and necrosis (at high concentration). Moreover, in zebrafish embryos MC-RR caused abnormalities such as loss of vascular integrity and hemorrhages (Zhao et al., 2011). In adults, the toxin accumulated in testes of zebrafish induced damage: widened intercellular junction, hydropic changes of mitochondria, and vacuoles (Zhao et al., 2012).

Analysis of microRNA expression and proteomic analysis of subcellular targets involved in toxicological mechanisms have been performed in zebrafish exposed to MC-RR (Zhao et al., 2011, 2012). Expression changes in miR-430 and miR-125 families indicated that MC-RR has a significant influence on regulation factors (such as p53) and on cellular processes during development of embryos. Moreover, the upregulation of miR-31 and downregulation of miR-126 may be related to the vascular defects of embryos induced by the toxin. MC-RR significantly reduced the expression of a number of proteins, and altered the expression of miRNAs involved in regulation of multiple signaling pathways, mainly including metabolism, cell signaling, protein synthesis, and cytoskeleton maintenance (Zhao et al., 2011). On the other

hand, proteins from zebrafish livers, that exhibited special interactions with the MC-RR, were screened using a chemical proteomics assay. After protein exposure to MC-RR purified from cyanobacterial blooms, 17 proteins were identified, which were involved in biological processes such as cytoskeleton assembly, proteolysis process, lipid transport, and oxidative stress (Tuo et al., 2016). In this study, the heat shock cognate 71 kDa protein (hspa8) was shown to be a chaperone protein that specifically interacts with MC-RR, and that is involved in many cellular processes, including ATPase function (Stricher et al., 2013). These reports suggest that hspa8 could be an earlier marker of these changes, and the inhibition of ATP synthesis plays a pivotal role in MC-RR-induced oxidative damage. The proteomic results in testes of zebrafish i.p. injected with MC-RR revealed for the first time that the toxin altered glycolysis metabolism, calcium ion binding, and proteins involved in cytoskeleton assembly (such as tubulin and keratin), and in oxidative damage (such as heat shock protein and aldehyde dehydrogenase) (Zhao et al., 2012). These authors indicated that MC-RR induced reproductive toxicity mainly by inducing oxidative stress.

In the same model, Xie et al. (2015) demonstrated that pure MC-RR might trigger growth delay in zebrafish larvae by inducing thyroid toxicity, with altered hypothalamic-pituitary-thyroid (HPT) axis-related gene expression and decreased T4 and T3 concentration. Moreover, the potential embryotoxic effects of MC-RR in early life-stages of *Xenopus laevis* were studied (Fischer and Dietrich, 2000). The results observed in this work were: 1) MC-RR needs to be taken up orally to exert any toxic effects, transchorional/transdermal absorption being minimal; 2) frog larvae were not susceptible to MC-RR (up to 5 days of development) at concentrations from 1 to 2000 μg/L; and 3) serine/threonine protein phosphatase-1 and −2A (PP) inhibition was observed after 96 h of exposure to 2000 μg/L.

Globally, it appears that fish are much more tolerant to MC than mammals (Xie et al., 2004; Malbrouck and Kestemont, 2006; Zhao et al., 2008). However, up to now, mammalian studies on the toxic effects of MC-RR have been very limited. Mice administrated with a single LD_{50} dose of MC-RR (235.4 μg/kg b.w.) showed increased serum cytosolic enzymes and depletion of hepatic GSH levels (Gupta et al., 2003). Zhao et al. (2008) demonstrated an increase of LPO in rabbits as a result of exposure to an extract containing mainly MC-RR, and an enhancement of SOD activity as a defensive response to the oxidative stress induced. The authors concluded that increased LPO levels could be involved in the ATPase inhibition observed in hepatocytes and cardiocytes, which might be partly responsible for the loss of mitochondrial membrane potential (MMP). Moreover, DNA fragmentation was observed in mice exposed to MC-RR, and this DNA damage occurred before necrotic liver damage (Gupta et al., 2003). In addition, histological changes, such as bronchial epithelial necrosis and hyperplasia, are the most relevant pulmonary lesions after MC-RR exposure (Gupta et al., 2003).

MC-RR also induced hepatic lesions such as congestion, hemorrhage, degeneration and vacuolation of hepatic parenchyma, and mitochondria vacuolation in mammals (Gupta et al., 2003; Zhao et al., 2008). In rabbits, liver mitochondrial damage was more prominent and rapid than damage induced in cardiocytes. Nevertheless, the mechanisms for such differences still remain unclear (Zhao et al., 2008). However, in kidneys of mice exposed to MC-RR the toxin did not cause pathological changes, regardless of the UA, BUN, CREA, Na+ , and K + levels in the sera (Zhong et al., 2017). This study reported that the water metabolism dysfunction in mice was attributed to serum renin–angiotensin system (RAS) activation. The results showed that changes in the liver serum enzymes, such as ALT, AST, and ALP, occurred a week earlier than those in the serum RAS index components, and these changes trigger the RAS cascade to lead to polydipsia and polyuria. In this regard, 100 mg/kg of aliskiren, a blocker of the RAS pathway, could efficiently alleviate these symptoms, suggesting that water metabolic dysfunction is caused by a RAS signaling mechanism and indicating that the ultimate origin is liver damage and not renal injury in mice.

In general, few in vivo toxicity studies focused on a comparative toxicity evaluation of MC variants have been performed. Chen et al. (2006) demonstrated that MC-RR and its variants had the lowest LD_{50} values in mice obtaining the following rank of toxicity: MC-RR < MC-WR < MC-FR < [D-Asp^3]MC-WR < < [D-Asp^3]MC-FR < MC-LR. This agrees with results previously reported by Stotts et al. (1993). The differences between hydrophilic and hydrophobic MC could result in changes in organotropism, toxicokinetics and bioaccumulation (Pia and Meriluoto, 2003).

With regard to in vivo toxic effects and taking into account the patterns of exposure to MCs, not only acute toxicity but also chronic toxicity is important. Only for MC-LR is there a toxicological reference value for subchronic exposure, and the WHO (1998) established a TDI of 0.04 μg/kg b.w. based on a NOAEL of 40 μg/kg b.w. per day, determined for liver pathology in mice. It has been suggested that this evaluation should be extended to all other variants, expressing it as MC-LR equivalents, but the validity of extrapolation from the acute toxicity ranking obtained via i.p. injection to other routes of exposure, and most importantly among MC congeners, to chronic toxicity remains to be demonstrated (Testai et al., 2016). Moreover, a review of the scientific literature found no information on the potential mutagenic effects of MC-RR in vivo, and its genotoxicity data are also very limited (Gupta el al, 2003). Other toxicological aspects, such as immunotoxicity or reproductive toxicity, are also insufficiently investigated with this particular congener, whereas data are available for MC-LR or cyanobacterial extracts (Chen et al., 2016; Lone et al., 2016).

5. Concluding remarks

Cyanobacterial blooms occur in the environment, and their toxicity is due to the production of cyanotoxins (a single one or a mixture) and other active compounds. As the variability of cyanotoxin production can be very high among different blooms, risk assessments are based not on mixtures (whole bloom) but on individual toxins. In this regard, MC-LR has been used as the standard cyanotoxin, and for the others a toxicity equivalent factor approach has been used. However, the toxicokinetic and toxic potency of the different congeners can vary. Therefore, to perform realistic risk assessments, specific MC congeners should be taken into account, it being necessary to characterize the hazard, i.e. to know the toxicological profile. In this regard, this review has focused on MC-RR, the cyanotoxin most extensively studied after MC-LR. The data compiled show that the occurrence of MC-RR in water is distributed worldwide and that its accumulation in food is possible. Moreover, MC-RR presents particular toxicokinetic aspects, and toxicity reports (both in vitro and in vivo studies) dealing with this congener are limited. The scarce number of toxicological studies on mammalian models (usually used for human toxicity interpolation), and particularly those related to chronic effects, is noteworthy. Further research is therefore required in order to determine the human and environmental risks due to MC-RR exposure.

Acknowledgments

This work was supported by the Spanish Ministerio de Economía y Competitividad (AGL2015–64558-R, MINECO/FEDER, UE). The authors are also grateful to the Spanish Ministerio de Economía y Competitividad for the grant FPI 432 (BES-2016–078773) awarded to Leticia Díez-Quijada Jiménez.

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